COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH

THE NEW ZEALAND JOURNAL OF SCIENCE AND TECHNOLOGY

A. AGRICULTURAL RESEARCH SECTION

VOLUME 31.—No. 2 AUGUST, 1949

Printed for the Department of Scientific and Industrial Research, Wellington,
by
Coulls Somerville Wilkie Ltd., Dunedin, N.Z.

October, 1950

REGISTERED AT G.P.O., WELLINGTON, AS A MAGAZINE

THE NEW ZEALAND

Journal of Science and Technology

VOLUME 31, No. 2, 1949

(AGRICULTURAL RESEARCH SECTION)

CONTENTS

PAGE

Studies in Monozygotic Cattle Twins, II. *John Hancock* 1

The Blackberry Mite (*Aceria essigi*). *Avice Hamilton* 42

A Recent Outbreak of Honey Poisoning, VII. *T. Palmer-Jones* and *E. P. White* 46

NOTICES

SUBSCRIPTIONS.—Subscriptions payable in advance. Series A, 10s. per annum; Series B, 10s. per annum; A and B, 15s. per annum. Single copies, 2s.; postage, 1d. Subscriptions should be paid by money-order, postal note, bank note or cheque, and should be forwarded to the Publications Officer, Department of Scientific and Industrial Research, P.O. Box 562, Dunedin. Cheques should be made payable to the Department of Scientific and Industrial Research, and when payable at banks other than in Dunedin should have exchange (6d.) added. Subscribers are requested to furnish their full names and addresses.

CORRESPONDENCE.—All communications relative to the Journal should be addressed to the Editor, Department of Scientific and Industrial Research, Wellington.

THE NEW ZEALAND
JOURNAL OF SCIENCE
AND
TECHNOLOGY

Editor: N. A. Marris, M.Sc., B.Com. (Assistant Editor: M. O'Connor, M.Sc.)
Department of Scientific and Industrial Research, Wellington

VOL. 31. SEC. A AUGUST 1949 NUMBER 2

STUDIES IN MONOZYGOTIC CATTLE TWINS: II. RECOGNITION OF MONOZYGOTIC TWINS

By JOHN HANCOCK, Ruakura Animal Research Station, Department of Agriculture, Hamilton

(*Received for publication 8 April 1949*)

Summary

(1) The usefulness of various criteria in the recognition of monozygotic cattle twins is discussed.

(2) While adopting the "similarity" method of diagnosis, the problem is approached from a new angle in that when a set of twins is examined, points of differences and not points of similarities are looked for. Correct diagnosis depends mainly on the elimination of dizygotic twins.

(3) Insofar as similarities are noted, only those concordant characters which have been shown to be of low frequency in the general population are considered.

(4) Frequency tables of various whorls and mouth pigmentation patterns in Jersey and Jersey-cross calves are presented.

(5) Simple rules for diagnosis are advanced.

(6) Evidence is presented of the accuracy of the diagnosis made in new-born calves, based on subsequent development when similarities and differences are more fully expressed.

(7) Attention is drawn throughout the paper to the extent and manner in which monozygotic twins may differ.

INTRODUCTION

ONE of the reasons for the slow progress in monozygotic cattle twin research has been the lack of a simple and accurate method of distinguishing between monozygotic and dizygotic like-sexed twins.

Kronacher (1) (2), first attempted to solve this problem and devised a system based on the hypothesis that if the members of a set of twins were very alike in a large number of morphological and physiological characters this fact could only be explained by assuming that the twins were of monozygotic origin.

Kronacher attempted to express the similarity or dissimilarity of a set of twins quantitatively. He allotted points on a 1-3 scale according to the degree of similarity shown by twins in a large number of characters. The result was expressed as a "similarity quotient" representing the average of the sum of the ratings for all the individual characters. The nearer 3 the value of this quotient approached, the greater was the probability of monozygocity. According to this system 35 sets of like-sexed twins were classified as follows:—

(A) 2 sets certainly monozygotic.
3 sets very probably monozygotic.

(B) 9 sets possibly monozygotic.

(C) 17 sets probably not monozygotic.
4 sets not monozygotic.

Haak (3) continuing Kronacher's work, discarded all physiological and endocrinological tests employed by the latter, but retained head and body measurements as criteria. Even these were considered largely as adjuncts to subjective judgment.

The similarity method was originated by workers on human twins. Newman and his collaborators (4) reported the results of investigations on more than 100 sets of twins. Their method of diagnosis consisted of a preliminary classification based on the degree of likeness of physical features, followed by a confirmatory test involving chiefly comparison of finger and palm-patterns. The preliminary tests which were wholly subjective gave correct diagnoses in 98 per cent. of cases.

Although workers in this field have been reluctant to admit the fact, it is difficult to escape the conclusion that no single test, nor any group of objective tests, has been devised whereby monozygotic twins may be distinguished from dizygotic twins with certainty.

Body measurements, which have been used in both the human and bovine species, have notably failed as quantitative tests of relative similarity. It is certainly true that if twins are arranged in order of increasing within-set differences, the top group contains only monozygotic twins and the lowest group only dizygotic twins. But there is a comparatively large middle group in which both types are mixed. In contrast to such ambiguous results, stands the relative ease and greater certainty whereby twins may be classified by mere eye appraisal. This situation probably depends on the following factors :

(1) The human eye is a measuring tool with which resemblances or differences in body form may be judged with great precision.

(2) Many of the characters in which monozygotic twins show the most striking resemblances cannot be evaluated objectively, *viz.* head form and coat colour.

(3) Accurate body measurements on the live animal are notoriously difficult to obtain because of a low repeatability. In consequence these have extremely large standard errors.

The general problems in connection with the location and collection of monozygotic twins in New Zealand have been discussed in a previous paper (5). To these must be added the problem of diagnosis, the difficulty of which is increased by the necessity for inspection of twins when they are only a few days old. Further, the time spent in actual diagnosis must be reduced to a matter of minutes as, in the height of the calving season, up to ten sets of twins may have to be inspected on widely separated farms in the course of a day. Under such conditions it will be evident that no objective tests are possible, and preliminary diagnosis has to depend entirely on eye judgment. As the calves are bought at the time of inspection at a price several times their slaughter value, a high degree of accuracy must be obtained to avoid excessive monetary loss involved in buying and rearing animals, which may later prove to be dizygotic. Thus, the usefulness of twins as experimental animals depends in no small degree on the availability of a rapid, precise method of distinguishing between monozygotic and dizygotic twins at an

early age. This is essential if the twins are to be used in experiments which involve differential treatment from an early age. Under such circumstances it is not possible to decide later whether differences developed in any subjectively or objectively measurable characteristics are due to the treatment imposed, or to the fact that the twins are not identical. In other words, it is not possible at a later stage, to carry out confirmatory tests to determine the zygocity of twins that have been subject to differential experimental treatment from birth.

This paper is divided into two parts. Part I contains a discussion in the light of the experience gained at Ruakura, of the relative merits of the various criteria of zygocity of new-born cattle twins used by previous workers and by the author. In addition, a description is given of the diagnostic procedure which has been adopted in the light of experience gained.

Part II marshals the available evidence to test correctness of the diagnosis. This evidence is based on observations which have been collected from twins selected as presumably monozygotic according to the method described in Part I, and subsequently reared in the same environment. It may be noted here that calves (especially Jersey and Jersey-cross animals) develop a number of characteristics at an early age of 3-4 months, which make diagnosis of a high degree of probability a relatively easy matter.

PART I. DIAGNOSIS OF ZYGOCITY IN NEW-BORN CALVES

MATERIAL

The method of collecting twins has been described in an earlier paper (5). In the requests to farmers to report like-sexed twins to the Ruakura Animal Research Station, the only point strongly stressed was that the calves should show no differences insofar as colour or shade of colour was concerned. There are good reasons to believe that this was the only character on which the inspected sample of like-sexed twins was selected by the farmers. Approximately 800 sets of like-sexed twins were inspected during the five seasons 1944-48.

CHARACTERS CONSIDERED

The following characters were considered and their relative value as an aid to diagnosis is discussed below.

(1) Size
(2) Body conformation
(3) Whorls
(4) Skin pigmentation
(5) Hair colour
(6) White markings
(7) Muzzle pattern
(8) Teats
(9) Anatomical abnormalities

(1) *Size.*

In humans, the size differences at birth of monozygotic twins equal those of dizygotic ones (4). Although no comparable data are known for cattle, the difference within sets of monozygotic twins are sometimes quite large. The greatest difference encountered was in the case of the unquestionably monozygotic twins T47 - T48 which differed by 17 lb. at 10 days of age, a deviation of 16 per cent. from their mean weight. Size is, therefore, not a very good criterion in zygocity of cattle twins,

but if the twins are to be used for critical experiments on growth, it is necessary to select only those monozygotic twins that show small weight differences since there are indications that such differences tend to persist (6). Detailed weights of twins diagnosed as identical will be reported in a subsequent paper on growth data.

(2) *Body conformation.*

Differences in body weight between new-born monozygotic twins may be due to a different degree of fatness or of muscular development, but observations suggest that they are mainly associated with skeletal size differences. Though such differences may exist within a monozygotic pair, they have no observable effect on relative body proportions so that on a conformation basis, the two animals will appear identical.

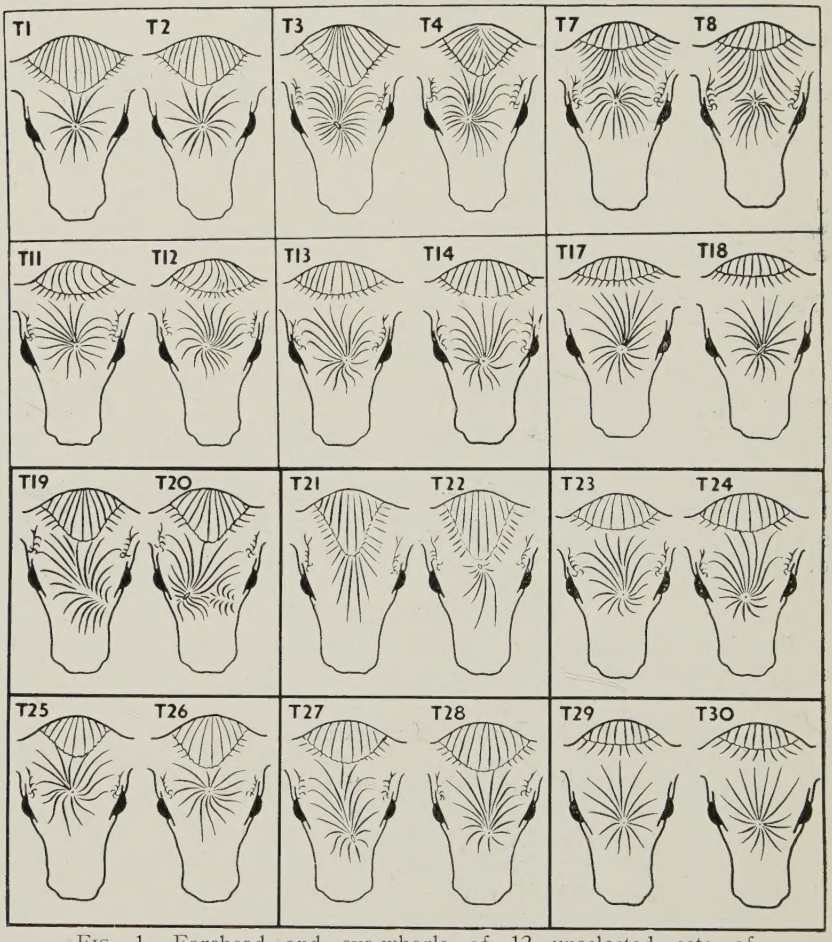


FIG. 1.—Forehead and eye-whorls of 12 unselected sets of monozygotic twins.

This condition is difficult to substantiate objectively since body measurements on the live calf are so inaccurate. However, it can be stated that two calves of different size which show similar body proportions, will seem more alike to the properly trained eye and mind than two other calves which are of the same size, but with differences in body proportions. This is the basis for a correct diagnosis of twins when the members of a set differ appreciably in size.

The position is easier if the calves of a set under inspection are of the same size. In such a case it is possible to compare directly by eye, the relative dimensions of various body parts of the two calves. It has been found that the heads are specially suitable for comparisons of this kind. Length and width of head, length and width of lower jaw, and circumference of muzzle, lend themselves to easy eye comparison. Less tangible characters such as the curvature of forehead, height of eye arches, size, shape and angle of muzzle and nostrils, and set and size of ears, also provide points for comparisons. Differences in these characters should be noted and mentally summed up. It may be stated as a general principle that even small differences in head form will be a strong indication that the calves are not monozygotic. To the trained stockman, accustomed to judge body form in animals, no difficulty is experienced in making decisions as to likeness or dissimilarity in head type.

(3) *Whorls.*

Whorls are most commonly present in cattle on the forehead, above the eyes, in the neck behind the poll, and on the midline of the back. It has been found that similarity or dissimilarity of whorls is not a very good criterion of zygocity in that a set of twins may show quite big differences in whorl pattern and yet may be monozygotic. Thus, in a sample of 90 sets of twins considered to be monozygotic, in eight cases one calf had a forehead whorl which was missing in the other;

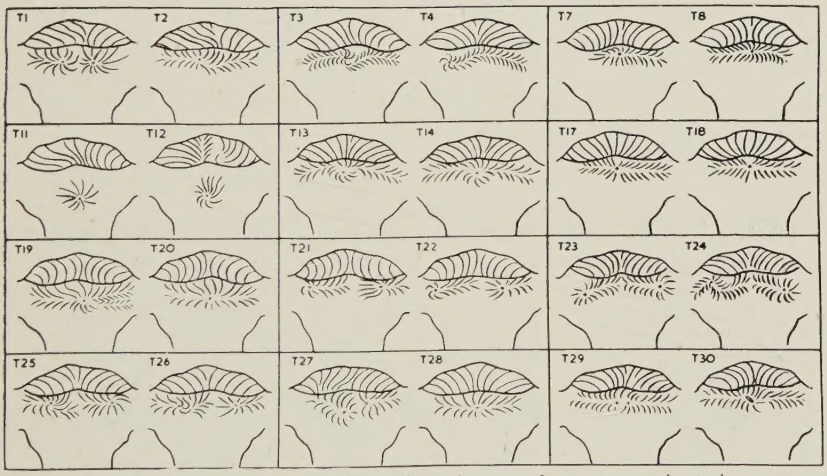


FIG. 2.—Poll whorls of 12 unselected sets of monozygotic twins.

in 16 cases the calves had a different number of whorls above the eyes; in 12 cases, one calf had two poll whorls whereas the other had only one. In addition it is important to emphasize that though major differences of the kind mentioned are fairly rare in monozygotic twins, whorls will always show at least some minor differences as to type, position or twist. The position may be appreciated better by studying Fig. 1, which depicts the forehead and eye whorls of an unselected sample of 12 sets of monozygotic twins. The poll whorls of the same twins are shown in Fig. 2. This representation of the whorls is somewhat diagramatic, but nevertheless gives a fair idea of the differences which can be expected. As additional evidence of the magnitude of differences which may occur, the head whorls of a two-headed calf are

shown in Fig. 3. This monster was brought to the notice of the author by Mr. Bonner, Inspector of Stock, Te Awamutu, in whose possession the mounted specimen remains. The heads were joined along the zygomatic arch. It has been suggested (1) that a monster of this type may be taken as a set of monozygotic twins which, because of an arrest in the splitting process, possess only one body with two heads. On this theory, such monsters provide a particularly useful illustration of the extent to which the whorls of a set of monozygotic twins may differ. The two heads show some striking resemblances, (the atypical whorls on the lower jaws) but also appreciable differences (position and shape of the forehead whorls).

Table V (Appendix I) compares the whorl pattern in 40 sets of twins considered to be monozygotic. An examination of this table shows that whereas most monozygotic twins have a similar whorl pattern, there are quite a number which show marked within-set differences. From this it is evident that if too much stress is laid on concordance in whorls,

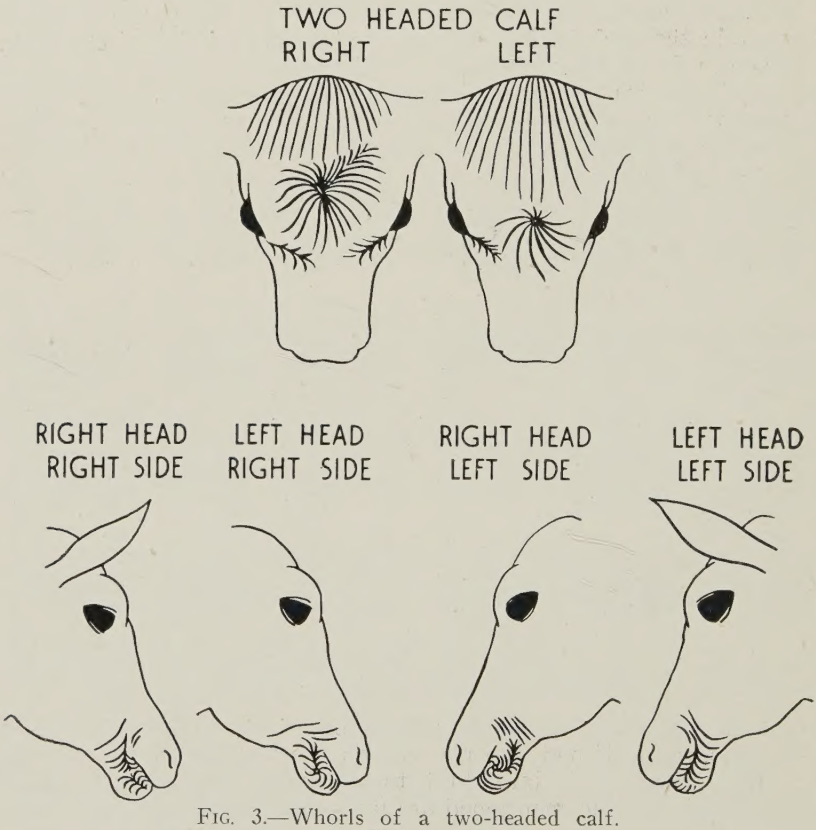


FIG. 3.—Whorls of a two-headed calf.

or if the inspected sample of twins is pre-selected so as to contain only sets showing high concordance in whorls, a great many sets of monozygotic twins will be missed.

In the course of the inspection of a large number of twins, it was observed that some types of whorl occurred less frequently than others, and it was thought that this fact might be utilized to increase the usefulness of whorls as a criterion of monozygotic twins. It is suggested that

if both members of a set of twins have a whorl which is of a rare type or in a rare position, this fact is better evidence of monozygocity than if they both had the same whorls but of a common kind.

To test this theory some exact information on the frequency of various whorl types was obtained from a sample of 400 calves randomly selected from the Horotiu Freezing Works. Frequency tables (see Table I) are presented for forehead whorls, eye whorls, poll whorls, and back whorls.

This special study was also considered desirable for two further reasons :—

(a) Whorls are easily recognizable unit characters and they are thus suited to subjective and objective comparison.

(b) Both Kronacher (1) and Bonnier (7) have stressed the importance of resemblance of whorls as an indication of monozygocity in cattle twins.

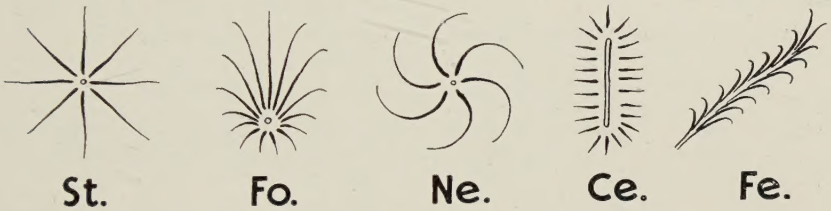


FIG. 4.—Diagrammatic representation of whorl types.

Forehead Whorls. Five different types of whorls may be distinguished fairly accurately. They have been designated—Star (S), nebula (N), fountain (Fo), centipede (Ce) and feather (Fe). These five types of whorls have been represented diagramatically in Fig. 4.

Table I shows the frequency of the various whorls in the sample of calves mentioned above.

TABLE I. FREQUENCY OF HEAD WHORL TYPES

	No.	Percentage.
Star	173	43.25
Nebula	81	20.25
Fountain	65	16.25
Centipede	12	3.00
Feather + Whorl	10	2.50
Feather from right eye	3	0.75
Feather from left eye	5	1.25
Feather from both eyes	5	1.25
Double Whorl	7	1.75
No Whorl	39	9.75
Total	400	100.00

The whorls were also classified according to their twist. These include only 341 calves as the remainder possessed whorl types which could not be classified this way. The results were as follows :—

Anticlockwise twist	198 or 58.1 per cent.
Clockwise twist	104 or 30.5 per cent.
No twist	39 or 11.4 per cent.

In view of the stress placed upon apparent mirror imaging in twist rotation as a test of monozygocity, the high incidences of both types must be noted. It will be clear that since there is a large chance that any two calves chosen at random will show reverse twist rotation, this characteristic is a very poor criterion of monozygocity.

The position of the forehead whorls can be determined with the aid of a co-ordinate system where a horizontal line running between the corners of the eyes represents the abscissa and a vertical line running from poll to muzzle dividing the head symmetrically into two halves, represents the ordinate. Fig. 5 shows the distribution of whorls of 341 calves. For obvious reasons the table does not include calves which have either two whorls, no whorls, or whorls of the feather type.

It may be noted, that the most common position of the forehead whorls is at the x=0 y=0 point, in that 83 calves of the 341 with one whorl only, had the whorl placed at this point. Seventy-seven of the calves had whorls below the abscissa and 128 above this line, 44 had the whorl on the right of the ordinate, and 115 left of this line.

Poll Whorls. The position, twist, and numbers of poll whorls have been combined into one table. The frequency of the various combinations are shown in Table II. It will be noted that marked differences exist.

TABLE II. FREQUENCY OF VARIOUS NUMBERS AND TWIST OF POLL WHORLS

No. of Whorls.	Position and Twist.*			Distribution.			
	Left. a n cl	Centre. a n cl	Right. a n cl	No. of Calves.	Part Total.	Per cent. of Calves.	Part total. (Per cent.)
1		+		68		17.0	
		+		61		15.25	
			+	16	145	4.0	36.25
1	+			50		12.5	
				14		3.5	
	+			13	77	3.25	19.25
1			+	3		0.75	
			+	3	6	0.75	1.5
2	+		+	78		19.5	
			+	21		5.25	
	+			18		4.5	
	+		+	17		4.25	
	+		+	4		1.0	
	+			4		1.0	
			+	3		0.75	
	+			3		0.75	
			+	1	149	0.25	37.25
2	+	+		3		0.75	
	+			2		0.5	
		+		2		0.5	
	+	+		1		0.25	
				1	9	0.25	2.25
2		+	+	1		0.25	
		+		1	2	0.25	0.5
2		+ +		1		0.25	
		+		4	5	1.0	1.25
2	+ +			1	1	0.25	0.25
3				6	6	1.5	1.5
				400	400	100.00	100.00

* a = anti-clockwise twist. n = no twist. cl = clockwise twist.

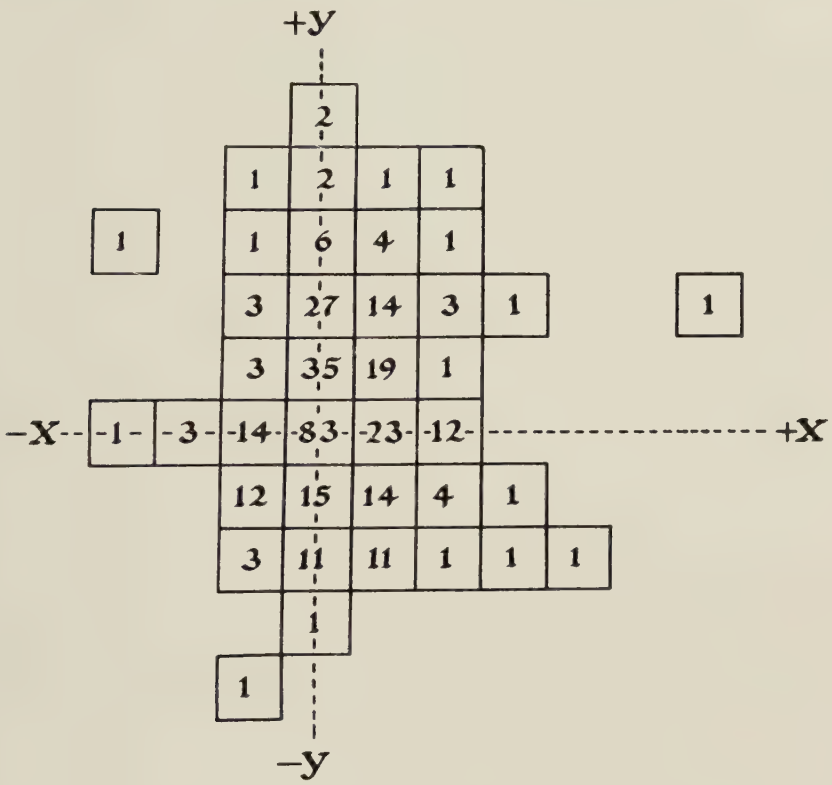


FIG. 5.—Frequency distribution of position of forehead whorls (see text).

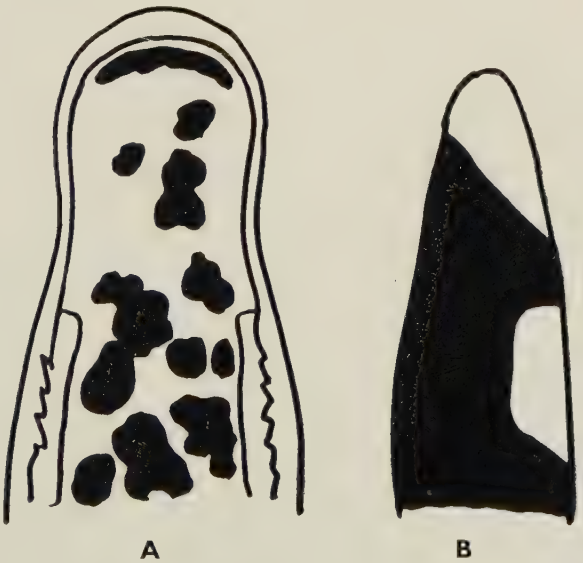


FIG. 6.—Typical pigmentation of (a) palate and (b) tongue.

TABLE III. FREQUENCY OF VARIOUS POSITIONS AND NUMBERS OF BACK WHORLS

	Distance from Top of Withers.																	
	−4–3	−2–1	+0	+1–2	+3–4	+5–6	+7–8	+9–10	+11–12	+13–14	+15–16	+17–18	+19–20	No Whorls.	Two Whorls.
No. of Calves.	2	2	51	8	11	52	72	86	70	5	5	4	1	1	30
Percentage	0.5	0.5	12.75	2.0	2.75	13.0	18.0	21.5	17.5	1.25	1.25	1.0	0.25	0.25	7.5

Back Whorls. The position of the back whorls is determined by measuring the distance from the whorl to the point where the shoulder blades meet. Whorls anterior to this point have been designated as "−", and when posterior as "+". Table III shows the frequency distribution of the position and the number of back whorls.

Eye Whorls. The frequency distribution of various combinations of eye whorls is shown in Table IV.

Table IV. Frequency of Various Eye Whorl Combinations

Right Eye.	Left Eye.	No. of Calves.	Per cent. of Total.
−	−	254	63.5
+	+	89	22.25
+		30	7.5
−	+	27	6.75
	Total	400	100.00

Symbol "+" and "−" indicates presence or absence respectively.

Examination of these data makes it quite clear that marked differences exist in the frequency of whorl types in all major positions and that certain types have a very low frequency.

On the basis of this fact it is argued that if the whorl pattern is the same in both members of a set, the likelihood that the twins are monozygotic judged on the whorls alone is greater if the whorls are of a type and in a position which show a low frequency in the general population than if the opposite is the case. To illustrate this, two sets of twins with the same within-set whorl formula may be considered.

	Forehead Position.	Whorl Type.	Eye Whorls.	Poll Whorls	Back Whorls.
Set 1	x=0 : y=0	S	Right eye − Left eye −	Right 1 a Left 1 cl	+10
Set 2	x=1 : y=2	Ce	Right eye + Left eye −	1 Right a	− 3

Tables I to IV show that the whorl formula of set 1 occur frequently whereas the whorls of set 2 are rare. Little importance may thus be attached to the resemblance of whorls in set 1, whereas set 2 probably could be judged monozygotic on grounds of whorl resemblance alone.

On this basis it is probable that set 18 with the rare forehead and eye whorls, set 37 with clockwise whorls left of the mid-line of the face and rarely placed back whorls, set 20 with no head whorls, rare poll whorls and double back whorls, could all be judged identical on whorls alone (Table V—Appendix I). In contrast, although the members of set 2 showed a marked similarity in whorl pattern of a very common type, it would be a risky porposition to assume monozygocity without other confirmatory tests.

(4) *Skin Pigmentation.*

Characteristic pigmentation patterns may be observed in Jersey and Jersey-cross calves on the bare or slightly hairy areas of the skin. Such areas are the muzzle, lips, palate and tongue, lower jaw, inside of ears, the ano-vulval region and the udder.

Muzzle and Lips. The colour of muzzle and lips ranges from almost black through greys to pink. In general the colour is darkest nearest the nostrils gradually becoming lighter towards the upper lip. Any

difference in the "colour gradient" of the nose or lips is a very strong indication that the twins are dizygotic. There are sometimes unpigmented patches on the nose or lips. Monozygotic twins may show discernible differences as to the extent and position of these unpigmented spots. Wholly unpigmented noses are fairly rare in the Jersey-cross cattle population so far examined in New Zealand.

Pigmentation of Mouth. Mouth colours in Jersey and Jersey-cross cattle vary from whole blue to whole pink through the range of intermediate stages. There are cattle with blue tongues and pink palates and cattle with the reverse combination. Spotted gums and tongues also occur frequently. The expression of the spotting factor is different on tongue and palate. The spots on the palate are small, round or slightly irregular, giving the area a mottled appearance (Fig. 6a), whereas the pigmented areas of the tongue are relatively large and unbroken (Fig. 6b). The same 400 calves described in connection with whorls were also examined as to colour of mouth. Table VI shows the frequency distribution of various patterns.

TABLE VI. THE DISTRIBUTION OF VARIOUSLY COLOURED MOUTHS

Palate—Per cent. Area Pink.	Tongue—Per cent. Area Pink.										
	0	10	20	30	40	50	60	70	80	90	100
0	166	3	–	1	1	2	1	1	–	–	12
10	15	–	–	1	–	1	–	–	–	–	4
20	2	–	–	–	1	–	–	–	–	1	2
30	3	–	–	–	1	–	–	–	–	–	3
40	4	–	–	–	–	–	–	–	–	–	4
50	11	–	–	–	1	2	–	–	–	–	3
60	14	–	–	–	–	–	–	–	–	–	–
70	3	–	–	–	–	–	–	–	–	–	–
80	3	–	–	1	2	–	–	–	–	–	1
90	22	–	–	1	–	3	1	–	–	–	14
100	22	2	1	4	–	3	1	–	–	3	54

It will be seen that over 40 per cent. of the calves had wholly pigmented tongues and palates and that approximately 13 per cent. were wholly pink or devoid of pigment on these surfaces. Most of the remainder had wholly pigmented tongues with partly pigmented palate. In this connection it may be pointed out that the spotted patterns on tongue and palate respectively probably depend on different genetical factors in that there were only very few individuals with both spotted tongue and palate.

From Table V (Appendix I) it will be observed that monozygotic twins show a fairly close resemblance in this character in the majority of cases. Colour of mouth is thus a useful adjunct to diagnosis as long as it is remembered that little notice should be taken of concordance if the calves show pigmentation of a common type.

It must also be noted that discordance in this character is not always a sign of dizygocity as some monozygotic twins show marked within-set differences (Table V Appendix I).

Pigmentation of Lower Jaw. The lower jaw often shows a characteristic pigmentation pattern, the most common consisting of a stripe of varying width of unpigmented skin running posteriorily from the lip, and flanked on both sides by pigmented areas. As monozygotic twins show no differences in this respect, this character may be regarded as a very useful criterion.

Ears. Jersey and Jersey-cross cattle often have a pigmented streak on the skin inside the ears which covers varying proportions of the lower part of the ear. A few common ear pigmentation patterns are shown in Fig. 7. No differences in the intensity or extent of this pigmentation has been observed in twins judged monozygotic. In many cases, it is possible to rule out monozygocity in a set of twins by comparison of the ear colour patterns alone.

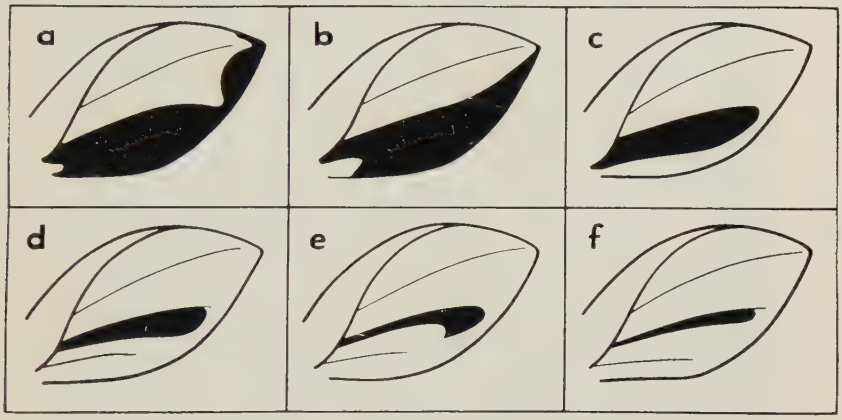


Fig. 7.—Common ear pigmentation patterns.

Pigmentation of the ano-vulvar region. Adult Jersey and Jersey-cross cattle often show a characteristic pigmentation pattern of this region, which varies in extent from a narrow pigmented strip to a patch covering the whole area and the surrounding skin, often extending far down the escutcheon. Differentiation is more definite with increasing age. In young calves the exact shape of this pattern is not easily discernible. It is, however, a certain sign of dizygocity if the members of a set of twins show appreciable differences in this respect at birth.

Pigmentation of Udder and Teats. The pigment of the udder, if present, differs from that of previously mentioned pigmented skin areas in that it is of a light brown colour instead of the usual blue-grey. Pigmentation of the udder is often found in Jersey-Ayrshire cross cattle. The pattern is irregular with the greatest number of pigmented areas generally on the teats. Monozygotic twins show differences as to extent and exact position of the pigmented areas of the udder but both twins must show either presence or absence of pigment if monozygocity is to be assumed.

The contention that in monozygotic twins, differences in area and pattern of pigmentation are permissible in the tongue, palate and udder but not in the ano-vulva region, ears and lower jaw would appear to involve an inconsistency. This apparent anomaly is explicable on the basis that in the latter areas pigmentation is anatomically symmetrical, while in the former, no symmetry occurs.

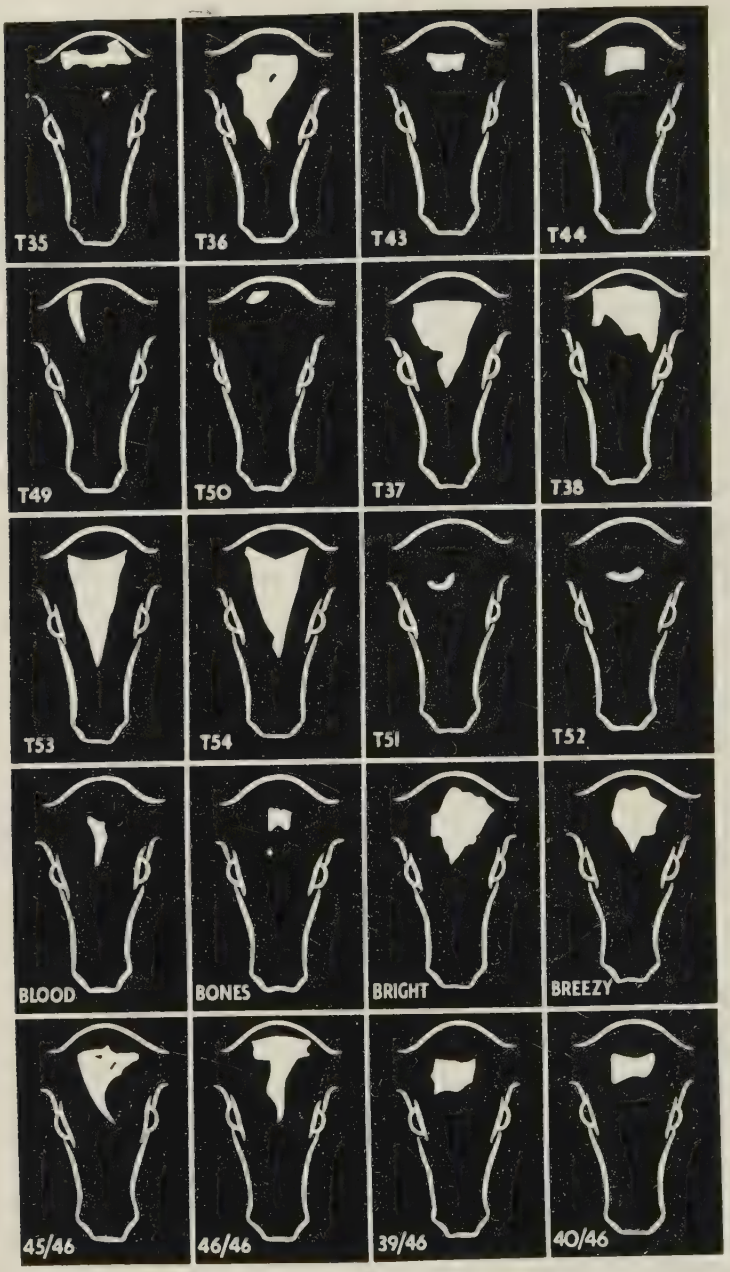


FIG. 8.—Forehead markings of ten sets of monozygotic twins.

(5) *Hair colour.*

There are two reasons why resemblance of hair colour should be regarded as a highly useful criterion of monozygocity in twins.

1. Hair colour in cattle is determined by a great number of independent genes (8). Especially in a heterogeneous population like the present cattle population of New Zealand, this lends the appearance

of a very great number of phenotypically different types. So numerous are these various colour types that a farmer knows each cow individually even in a large herd mainly by the colour differences shown.

2. Colour of hair is a strongly inherited character, influenced only to a small degree by environmental factors. This applies, however, to a lesser degree to special colour factors as (a) various white spotting factors, (b) factor for " brindle " colour, (c) factor for " roan " colour all of which are probably influenced by accidents in prenatal development. But the Jersey colour pattern ranging from almost white-grey or fawn-brown to black, seems to be so little influenced, at least in early life, by other than genetical factors that any difference in this pattern, however small, is strong evidence that the twins are dizygotic. The usefulness of colour as a criterion is, however, to a certain extent minimized by the fact that the adult colour pattern is almost totally obscured by the birth coat which, in Jersey and Jersey-cross calves, generally is of a uniform fawn colour as far as the general impression is concerned. In spite of this, it is possible to differentiate by examining the colour gradient from base to tip of the hair of the birth coat. This is done over various parts of the body by running the hand against the lie of the hair in a good light. It is possible to detect even quite small differences in colour in this way. It is of interest to note that trained observers can make a fairly accurate prediction in this manner of the adult colour pattern and shade in calves that are extremely alike in their visible birth coat.

The colours of some hair growths, such as eye lashes, ear fringes and tail swish, are not obscured by birth-coat colour and are therefore particularly useful for comparison. It is often possible to discard a set of new-born twins as dizygotic by an examination of the eye lashes alone.

(6) *White markings.*

It has previously been mentioned that the white colour markings are relatively weakly inherited and, apart from the need for a general similarity in pattern, are not a good criteria of zygocity. However, it has been claimed (9) that the forehead markings are inherited independently, and that the expression of this factor is not so easily influenced by prenatal environment as the factors which determine other types of white markings.

To show the range of differences occurring in monozygotic twins in this respect the forehead markings of eight sets of monozygotic twins are shown in Fig. 8. These have been drawn to scale from actual measurements.

It appears that the differences shown in this respect are of the same relative magnitude as the differences in markings on the rest of the body. See also colour prints in Part II. Forehead markings are thus no better criteria of zygocity than other white spots.

(7) *Muzzle pattern.*

In view of the short time available for inspection, and technical difficulties involved in taking good prints in the field, no muzzle prints are taken at the time of the first inspection. However, the muzzle patterns are examined by eye and the type of patterns are compared without any attempt to analyse detailed differences. When it is realized that only three or four classes of patterns occur in cattle (1) (10) and that

monozygotic twins may show quite appreciable differences as to the details, it can be concluded that nose patterns are of little value as evidence of zygocity in twin calves under the conditions of twin collection in New Zealand. See also Part II.

FIG. 9.—Monster, consisting of only a deformed head, born as a co-triplet to a set of normal monozygotic twins.

(8) *Teats.*

Because it has been shown (11) that the presence of supernumerary teats in sheep depend at least partly on inherited factors, it was thought that monozygotic cattle twins should show a high degree of concordance in this respect. However, an examination of 62 sets of monozygotic heifer twins showed that this was not the case.

TABLE VII. THE NUMBERS AND POSITIONS OF SUPERNUMERARY TEATS

	Twin 1.		Twin 2.	
No. of Sets.	Left.	Right.	Left.	Right.
26	—	—	—	—
5	+	+	+	+
3	—	+	—	+
1	+	—	+	-
2	—	+	+	—
2	—	—	+	-
2	—	—	+	+
8	—	—	—	+
4	+	—	+	+
4	—	+	+	+
5	Teat between fore and hind teats.		Not present.	
62				

Thus 35 or almost 60 per cent. of all sets showed concordance, two sets showed mirror imaging, and 25 showed discordance. Ninety per cent. of concordant sets were however of the type, — —, — —, that is, both calves lacked supernumerary teats. As this is a very common trait in dairy cows it is concluded that the teats do not provide an aid in the diagnosis of zygocity of cattle twins. This conclusion is in agreement with Kronacher's (1) observation (p. 411), ". . Aber

auch ganz bestimmte Korperliche Einzelheiten ersheinen nicht spezifisch erblich bedingt zu sein, wie z.B. der Besitz und Sitz von uberzahligen Zitzen am Euter. . ."*

(9) *Anatomical abnormalities.*

Several types of anatomical abnormalities have been observed in monozygotic twins. Some of these have been present already at birth, others have become apparent when the animals have grown up. Contrary to expectation, an abnormality generally occurs only in one member of a set, the other being normal. Some of the deformities present at birth may be due to the splitting process. An extreme case is shown in Fig. 9. This monster was a co-triplet to a set of normal monozygotic twins. Less extreme but still quite pronounced cases are listed below. Anatomical descriptions will be given in a separate paper.

FIG. 10.—The monozygotic twins T27 and T28. Note the deformed tail of T28.

1. Deformity present at birth.
   (a) Tail deformities. In four cases some deformity of the tail have been found. Fig. 10 represents the most extreme case.
   (b) Deformity of ears (Fig. 11).
   (c) Deformity of feet (Fig. 12).
2. Deformity, presence of which at birth uncertain.
   (a) Asymmetrical udder deformities. During routine examination of udder development Wallace (12) found two sets of monozygotic twins in which one member of each lacked one mammary gland. The animals were examined first when three months old.

* "but also specific anatomical traits do not seem to be specifically determined by genetical factors as for instance the presence or absence of supernumerary teats on the udder."

FIG. 11.—The monozygotic twins 21/48 and 22/48. Note the deformed ears of 21/48.

FIG. 12.—The forefeet of the monozygotic twins T73 and T74. Note the lack of dew claws in T73.

(b) Symmetrical udder deformities. On the owner's request a set of monozygotic $2\frac{1}{2}$-year-old twins were examined by the author. It was found that both members had non-functional fore-quarters. At the time of inspection the animals were in a fairly advanced stage of lactation but the owner claimed that the quarters concerned had

FIG. 13.—The monozygotic twins T61 and T62. Both have undershot jaws but the deformity is more pronounced in T62.

FIG. 14.—Heads of the monozygotic twins 465 and 466. Note the twisted face of 466.

never produced milk. As the animals could not be acquired it was not possible to ascertain the exact nature of the deformity. This is the only known case in which both members showed a deformity.

(c) Undershot jaw. Fig. 13 shows a set of monozygotic twins of which both members have undershot jaws but the deformity is decidedly more pronounced in one (T62).

3. Deformity not present at birth.

   (a) Twisted face. Both members of the set shown in Fig. 14 had straight faces when they were born but as they grew up one member developed a progressively twisted face while its co-twin remained normal.

All the twins reported here were easily diagnosed as monozygotic, in spite of the differences they showed in anatomical development. It is rather important to recognize that such differences are of no value in the diagnosis of twins, otherwise some valuable animals may be lost.

RESULTS AND CONCLUSIONS

As has been explained previously it is a relatively easy matter to diagnose twins correctly when they have lost their birth coats. To evaluate the success obtained by the methods outlined in this paper the classification of the twins at a very early age is, therefore, compared with a re-classification of the twins when the calves reached 4-5 months of age.

TABLE VIII. PRELIMINARY AND FINAL CLASSIFICATION OF TWINS

Year.	Monozygotic on Preliminary Classification.	Final Classification.		Doubtful on Preliminary Classification.	Final Classification.	
		Monozy.	Dizy.		Monozy.	Dizy.
1944	17	16	1	3	—	3
1945	37	34	3	1	—	1
1946	41	41	—	—	—	—
Total	95	91	4	4	—	4

Table VIII shows that in only four cases out of 95 classified as monozygotic, the diagnosis at an early age proved to be incorrect. All four sets which first were classified as doubtful turned out to be dizygotic. These findings indicated that (a) the method of diagnosis described in this paper leads to highly accurate results, and (b) very few, if any, monozygotic twins have been incorrectly diagnosed as dizygotic, since all borderline cases proved dizygotes.

In this connection it is of interest to describe in some detail those 4 sets of dizygotic twins which were incorrectly diagnosed as monozygotic, in order to show, if possible, the circumstances which led to the wrong conclusions.

Set (1). Apart from some white markings on brisket, belly and udder, these calves were jet-black in colour. The markings were very similar in both calves.

Set (2). These calves were fawn-coloured with white markings which were very similar in both calves. The twins were received by train after a great many difficulties and were 3-4 weeks old on arrival. They had been judged monozygotic by a field officer who had had some previous experience of diagnosis of twins.

Set (3). These twins also arrived by train and were 3 weeks old on arrival. They were of a very dark, almost black colour.

Set (4). The calves of this set were of light fawn colour with no noticeable colour graduations on any part of the body.

As the calves grew up it became evident that they were of dizygotic origin. Sets (2), (3), and (4) showed small but obvious colour differences and also differences in conformation. Both calves of Set (1) remained jet-black but showed such differences in head and body shape that it was impossible to assume that they were monozygotic.

It will be noted that from a diagnostic point of view all these four sets had one characteristic in common, the within-set colour differences were very small. This was probably the main reason why these sets were diagnosed incorrectly. In the case of the sets (3) and (4) however, there were additional features of more subtle nature which probably influenced judgement.

(a) Both sets were relatively old when bought which meant that the Station had to pay a much higher price for them than for most twins. It thus became particularly desirable that these twins should prove to be monozygotic.

(b) They arrived by train and as the author had the pre-conceived idea that such twins were more likely to be monozygotic than if they had been located by other methods (5), it is probable that they did not receive as thorough an inspection on arrival as otherwise would have been the case.

(c) The very similar white markings probably also tended to make the inspection of other points less thorough.

(d) It is likely that undue notice was taken of the opinion of the original observer who had diagnosed the twins as monozygotic.

Rules in diagnosis.

This discussion indicates that some of the difficulties in correct diagnosis of twins are of a psychological nature, and it is now possible to formulate a number of rules which should go some way towards making the task easier.

1. When for any reason the wish to find that a certain set of twins is monozygotic is stronger than normal, powers of discrimination greater than usual must be exercised.

2. When a fair standard of judging ability has been reached, little or no notice should be taken of the opinion of others, especially of an expressed opinion that the set under consideration is monozygotic.

3. Although the method of diagnosis propounded in this paper is essentially the "similarity" method of other workers in the field, the problem has been approached from a new angle. It has been repeatedly stressed that when a set of twins is examined, points of differences and not points of similarities should be looked for. This is because any two calves superficially at least, have so many traits in common, that it is easy to be carried away by wishful thinking into believing that almost any two calves of the same breed are monozygotic. However, if a number of calves are examined in a critical frame of mind, it will soon become apparent that in spite of resemblances all calves may show, they may also differ in a number of traits which will make them stand out as individuals, each one different from all other calves in the herd. This condition is the major factor underlying the successful diagnosis of twins expounded in this paper.

Briefly, the method adopted is as follows: The calves are held closely together with their heads in the same direction and each point of comparison considered separately. Differences in nose pattern, whorls, white markings, mouth, muzzle and ear pigmentations, shape

of head and coat colour are mentally noted. Special weight is given to differences in the four last mentioned characters as any differences noted here are considered a certain sign that the calves are dizygotic. In so far as any similarities are noted, only those concordant characters which have been shown to be of low frequency in the general population are considered. When all differences have been observed and mentally summed up, it is easy to form a final judgment on the question—are the differences of such a kind and their sum small enough to warrant the assumption that the twins are monozygotic ?

To check on the usefulness of various criteria the following test was carried out. Since the process is essentially one of elimination, 100 sets of dizygotic twins were classified according to the number and kind of criteria on which they could be diagnosed as definitely dizygotic. The criteria used and percentage of cases which could be diagnosed correctly on each one of these are shown below.

Hair colour	91 per cent.
Muzzle shape	91 ,,
Shape of head	72 ,,
Shape of lower jaw	72 ,,
Body conformation	52 ,,
Colour of eyelashes	39 ,,
Whorls	39 ,,
Pigmentation inside ears	36 ,,
Length of tail	25 ,,

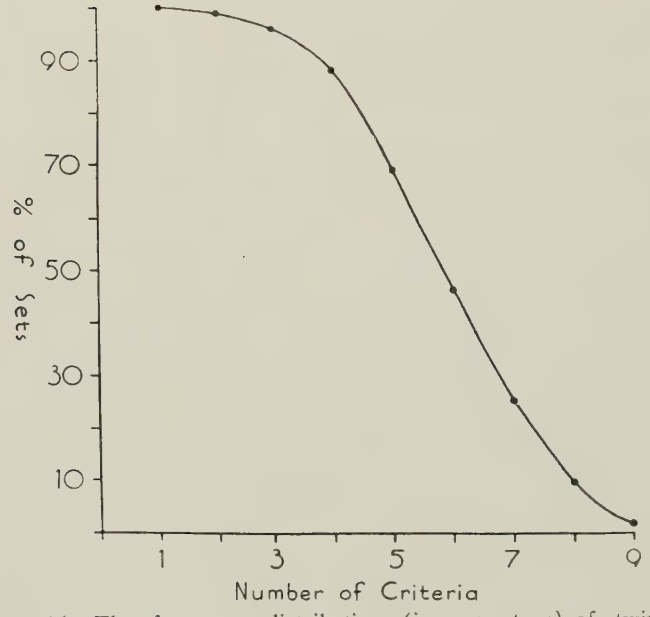


FIG. 15.—The frequency distribution (in percentage) of twins which could be diagnosed as dizygotic on various numbers of criteria.

It will be noted that only 9 out of the 100 sets could not be diagnosed as dizygotic on colour of hair. The majority of these sets were either white (2 sets), black and white (1 set), uniformly red (2 sets), or red and white (1 set). The remainder (3 sets) were of light fawn colour.

The shape of the muzzle was an equally good criterion but the others were also very useful. Fifty-six per cent. of the sets could actually be diagnosed as dizygotic on differences of either colour of eyelashes or pigmentation inside the ears, or both.

The distribution of sets which could be diagnosed as dizygotic on various numbers of criteria are shown in Fig. 15 based on an analyses of 100 sets.

It will be noted that 96 per cent. of all dizygotic twins differ in at least three criteria; that 50 per cent. differ in at least six criteria. Under these circumstances, it is easy to understand that most dizygotic twins can be recognized in a mere glance by a trained observer.

PART II. CONFIRMATORY TESTS AND EVIDENCE OF MONOZYGOCITY

Evidence for the accuracy of the foregoing method of diagnosing zygocity in new-born cattle twins rests as a whole on the thesis that some like-sexed twins are so very and so unusually similar in a great many characters that this fact can be explained only by assuming that they are of monozygotic origin.

As similarity is only a relative concept the degree of similarity within presumably monozygotic sets ideally should be compared with that occurring within dizygotic twins. This procedure has not been possible except in the case of whorls, because dizygotic twins for practical reasons could not be retained in sufficient numbers. The results are, therefore, expressed mainly on the basis of " within " and " between " sets comparisons of presumably monozygotic twins. This is, however, not a very serious failing since evidence will be presented later showing that New Zealand dairy stock is rather heterogeneous in respect of some of the most important characters considered. This ensures that even closely related individuals such as dizygotic twins show differences which are sufficiently marked in most cases to set them apart from monozygotic pairs.

Characters considered in the actual process of diagnosis described in the first part of this paper, are re-examined here in light of the evidence they are capable of yielding from further tests which cannot be carried out at the time of the initial diagnosis. Other characters which are not fully developed in new-born calves are also considered.

It must be emphasized that it is extremely important to determine the extent to which monozygotic twins may differ. Otherwise, many valuable animals may be discarded.

Characters Considered for Confirmatory Tests

In carrying out confirmatory tests for monozygocity the following characters were considered :

(1) Whorl pattern.
(2) Escutcheon pattern.
(3) Muzzle prints.
(4) Coat colour.
(5) Red cell factors.

(1) *Whorl pattern.*

In Part I of this paper it was shown that the whorl patterns were not a good criteria of zygocity of a set of twins in that some monozygotic twins showed considerable differences, while some dizygotic twins were very similar. In so far as the expression of the whorl pattern

depends on genetical factors, it is, however, logical to assume that the whorl patterns are, on an average, more similar within monozygotic twins than within dizygotic ones. The existence of such a relationship between the two samples of twins designated by the author as mono- and di-zygotic would, therefore, tend to support the claims that the diagnosis has on the whole been correct.

In order to compare the similarities of whorl pattern within a number of twin sets, it is necessary to devise a method whereby degrees of concordance can be measured. Kronacher (1) attempted to do this by assigning each set a value according to the following scale.

Whorl pattern very similar	3
Whorl pattern not very similar	2
Whorl pattern not similar	1

This system of scoring, based as it is on subjective judgments, is open to the criticism that the results may easily be biased by preconceived opinions about the zygocity of the twins. In the present investigations this difficulty was avoided by allotting points for degree of similarity on a scale which was based on such easily distinguishable features as the type and twist of the whorls, and on their relative positions. As the similarity of mouth pigmentation can be measured quantitatively this characteristic was also included. The points were scored according to the scale shown in Table IX.

TABLE IX.

Position of forehead whorl	Within the same $\frac{1}{4}$ cm.^{2}	3 points
	Not within the same $\frac{1}{4}$ cm.^{2} but within the same 1 cm.^{2}	2 points
	Outside limit.	1 point
Type and twist of forehead whorl	The same type and twist.	3 points
	N. in one member and S. in the other, any twist.	2 points
	Different type.	1 point
Poll Whorls	The same number of whorls and twist or asymetric reversal of twist if forehead whorl shows this characteristic.	3 points
	Same number but different twist.	2 points
	All other cases.	1 point
Back Whorls	Same number, position and twist or asymetric reversal if forehead whorl shows this characteristic.	3 points
	Within 2 cm. any twist	2 points
	Other combinations	1 point
Eye Whorls	The same number and position or $\pm \mp$ if forehead whorl shows asymetric reversal.	3 points
	$\pm \mp$ if forehead whorl does not show asymetric reversal.	2 points
	Other combinations.	1 point
Mouth Pigmentation	Same pigmentation of tongue and palate.	3 points
	Average difference in pigmentation of tongue and palate 10 per cent. or less.	2 points
	Greater differences	1 point

Methods used in measuring the above characters have been described in Part I.

It will be noted that on this system, the total maximun points are 18 and the total minimum points 6, while the maximum and minimum

average points are respectively 3 and 1.

Forty sets each of presumably monozygotic and dizygotic twins selected at random were scored according to this scale. For the sake of comparison the records of 80 single born calves paired at random were treated in a similar manner. The results are shown in Table X which gives the average points scored by the three classes of animals for each character and the total average points for all the characters combined.

TABLE X. WHORL SIMILARITY QUOTIENTS

	Monozygotic Twins.	Dizygotic Twins.	Calves Paired at Random.
Position of forehead whorls	2.22	1.67	1.34
Type and twist of forehead whorls	2.47	2.07	1.37
Poll whorls	2.59	1.84	1.46
Back Whorls	2.28	1.56	1.40
Eye Whorls	2.61	2.09	2.07
Mouth Pigmentation	2.73	2.09	1.51
Total	14.90	11.32	9.05
Average	2.48	1.89	1.52

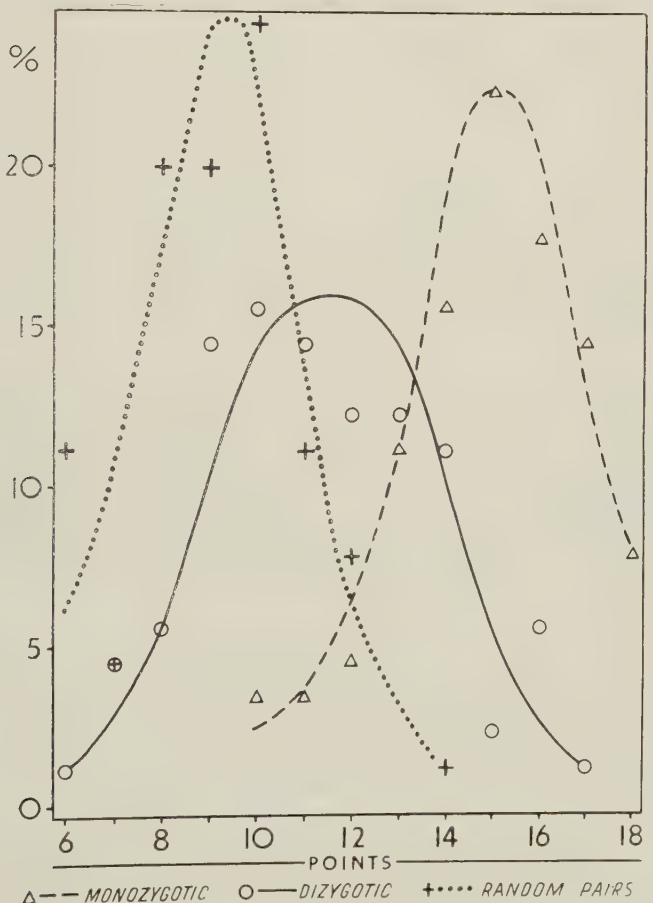


FIG. 16.—Percentage frequency distribution of similarity quotient in three samples of calves (see text).

It will be seen that the average score of the monozygotic twins for all characters was approximately 1.0 point higher than that of the calves paired at random with the score of the dizygotic twins intermediate. Furthermore, the scores for each of the characters were highest for the monozygotic twins with the scores for the dizygotic twins ranking second. However, in the case of the eye whorls and back whorls, the differences between the dizygotic twins and the calves paired at random appear to be insignificant.

It can, therefore, be stated that the preliminary division of the inspected twins into two groups, one designated as monozygotic and the other as dizygotic, has been justified on the ground that the former show much greater within-set similarities than the latter. The probability of the three samples of paired animals belonging to three distinct populations is clearly demonstrated in Fig. 16.

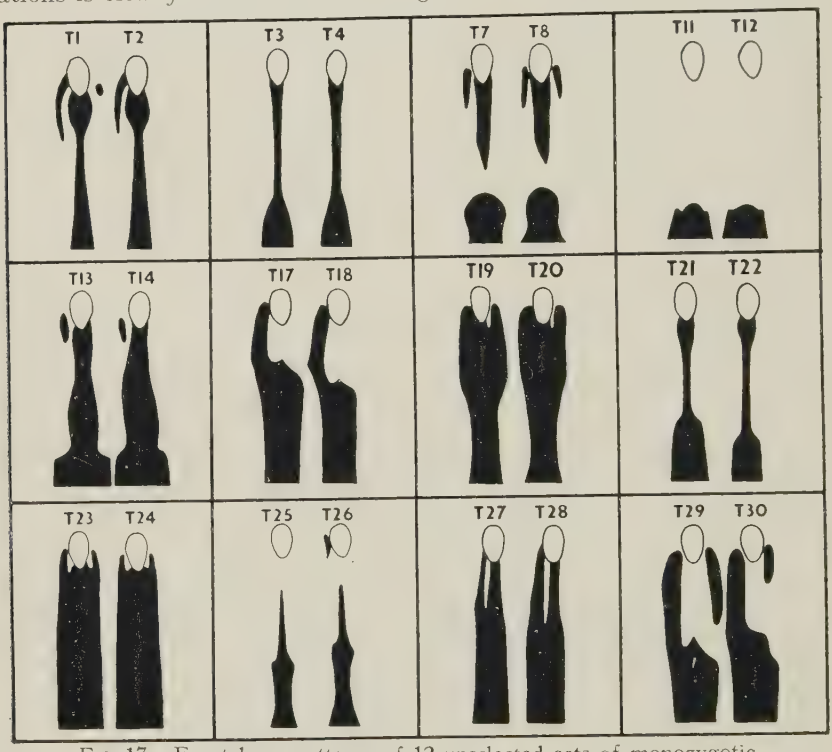


FIG. 17.—Escutcheon patterns of 12 unselected sets of monozygotic twins.

(2) *Escutcheon pattern.*

Once the birth coat is lost the escutcheon of cattle is clearly defined. Observations on a number of twin sets which had been diagnosed as presumably monozygotic at an early age, made it evident that the escutcheon patterns were extremely similar within sets, whereas the differences between sets were in most cases very pronounced. As it is not possible to measure the degree of similarity of a character of this nature, the only way to demonstrate the correctness of the above statements is per medium of a pictorial record. In Fig. 17 the escutcheon patterns are presented for 12 sets of monozygotic twins selected at random. The patterns are drawn to scale from actual measurements

on the animals. Even a cursory examination of these diagrams shows that the escutcheon pattern of an animal matches almost perfectly that of its co-twin and further, that unrelated animals display a very wide variety of easily distinguishable patterns. The combination of these two facts makes it possible to conclude that the similarity of the escutcheon patterns within each of the 12 sets of twins may be taken as contributory evidence that these twins were really of monozygotic origin.

The similarity of escutcheon patterns obviously cannot be used as an early criterion of zygocity, as this character is not recognizable in calves as young as those generally inspected. However, as the relative shape of the escutcheon pattern does not change even under very variable environmental conditions, this character provides a very useful check on previous early diagnosis, even if the members of a set have been exposed to different environments from an early age. This fact can be of special importance in certain classes of experimental work. Similar close resemblances in escutcheon patterns have been noted in all of the 170 sets of twins at Ruakura deemed monozygotic.

(3) *Muzzle prints.*

It has been shown in humans that monozygotic and dizygotic twins can be distinguished with a high degree of certainty by a comparison of the within-set similarities of palm and finger patterns. The success thus obtained is due to the circumstances that : (a) these characters are strongly inherited, and (b) an almost endless variety of patterns occur which can be defined with mathematical precision. Previous workers on cattle twins (1) and (7) have attempted to use the muzzle prints in a similar manner and have claimed some measure of success. Under the conditions of twin collection in New Zealand the muzzle patterns can only be useful as a confirmatory test since taking muzzle prints presents too great technical difficulties, considering the short space of time available at the first examination on which a decision has to be made as to whether or not the animals should be acquired.

The muzzle prints of 12 sets presumed monozygotic twins are presented in Fig. 18. These prints were obtained when the animals were approximately a year old. On a casual examination it would appear that the muzzle patterns are quite useful as a criterion on monozygocity, since the within-set differences on the whole are small, while there are some quite pronounced differences at least between some sets. However, a more critical examination reveals that in some cases the differences within sets are of such magnitude that the pattern of an animal is better matched by that of an unrelated animal than by the pattern of its co-twin. This applies, for instance, to T22 and T20. The degree of dissimilarity possible within monozygotic twins is also illustrated in Fig. 19 which shows the muzzle prints of a two-headed calf. It can be seen that the two patterns show differences of quite the same magnitude as shown by the worst matched sets in Fig. 18. The similarity between some unrelated animals are, on the other hand, so great that they may well belong to the same set. T7, T19, T23, and T25 may be members of a set of monozygotic quadruplets, as far as muzzle patterns are concerned. As the patterns of unrelated animals may be so similar, it is logical to assume that the pattern of a set of dizygotic twins will frequently show equally great, if not greater, similarities. This is borne out by an examination of the muzzle prints in

Fig. 20 which represent the patterns of the only available set of definitely dizygotic twins. It seems that the within-set similarities of these animals are as great as shown by any of the 12 sets of presumably monozygotic twins which have been previously discussed. The weakness of the muzzle patterns as a criterion of zygocity of a twin set depends thus on the two facts that (a) monozygocity cannot be ruled out even if the patterns show some important dissimilarities in details, and (b) there are altogether only a few easily distinguishable patterns which increases the likelihood that dizygotic twins will possess similar patterns.

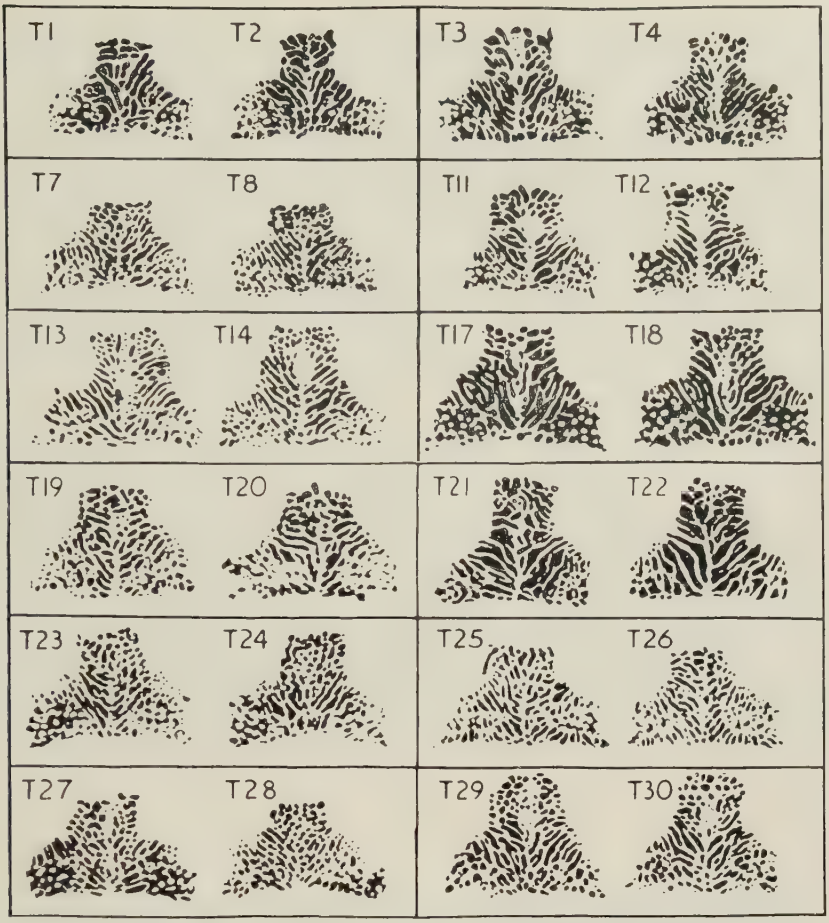
(4) *Coat colour.*

The usefulness of coat colour as a check on early diagnosis depends on the fact that a great range of phenotypes exists which can be distinguished with ease and accuracy on animals which have shed their birth-coats. As already mentioned, this applies particularly to the present cattle population in New Zealand, which, although predominantly of Jersey type, shows innumerable traces of its Shorthorn ancestry and of recent sporadic top-crosses with Friesians and Ayrshires.

Observations of approximately 170 sets of twins which had been diagnosed as presumably monozygotic at an early age and which had subsequently been reared under similar conditions, indicate that such animals show remarkably small within-set differences in respect of coat colour, with the exception of the following three colour patterns: roan, brindle, and white spotting. Otherwise this rule applies not only to the main hair colour such as the various shades of red, but also to the smallest details of the pattern produced by variously pigmented hairs dispersed throughout the coat.

The patterns referred to are those common to the majority of Jersey cattle. They consist of dark or black patches set against light areas of red, brown, fawn, or grey. The extent of the dark areas varies greatly from one animal to another. In the one extreme blackish hairs appear only as a smudge on the face, whereas in the other extreme jet-black hair covers the whole coat except for a small area below the horns, and on a strip along the back. Between these extremes a great number of intermediate stages occur, often characterized by intricate patterns on the faces, foreheads, and the anterior part of the fore-legs. On the light areas many different colours exist either side by side thus forming a distinct pattern, or blend into a diffused one. It can be readily understood that very numerous patterns can be evolved by a combination of various dark and light coloured hairs. In fact, so numerous are the possible combinations that, except for monozygotic twins, it would be very difficult, if not impossible, to find in a finite population of Jersey cattle, two animals with exactly the same colour pattern. This is the reason why 95 per cent. of the dizygotic twins inspected at birth showed within-set colour differences even at an age when colour was only poorly developed. Monozygotic twins differ sharply from both unrelated paired animals and dizygotic twins in that they show no within-set dissimilarities as far as the Jersey pattern is concerned, provided they have been reared under similar conditions.

An attempt to demonstrate the truth of this statement has been made by the provision of Kodachrome colour prints of a number of monozygotic twins reared at Ruakura (Figs. 21a and 21b). In reproduction, many of the finer details of coat colour have been lost, but the prints



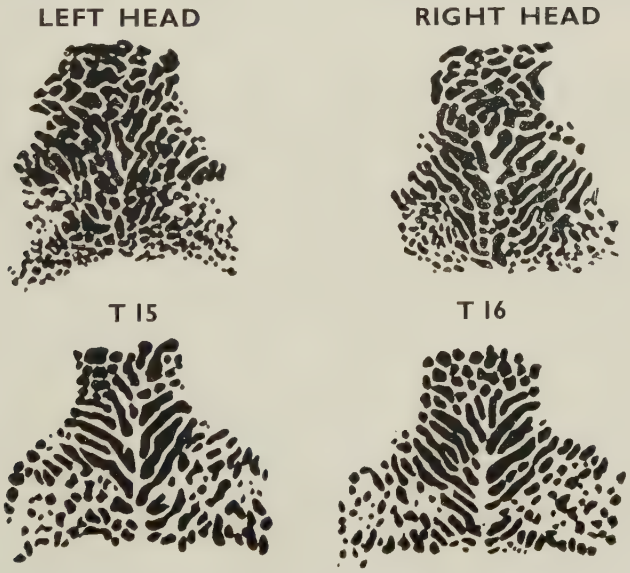


FIG. 18.—Muzzle prints of 12 unselected sets of monozygotic twins.

FIG. 19.—Muzzle prints of two-headed calf. Note the marked dissimilarities.

FIG. 20.—Muzzle prints of a dizygotic set of twins. Note the similarity in general pattern.

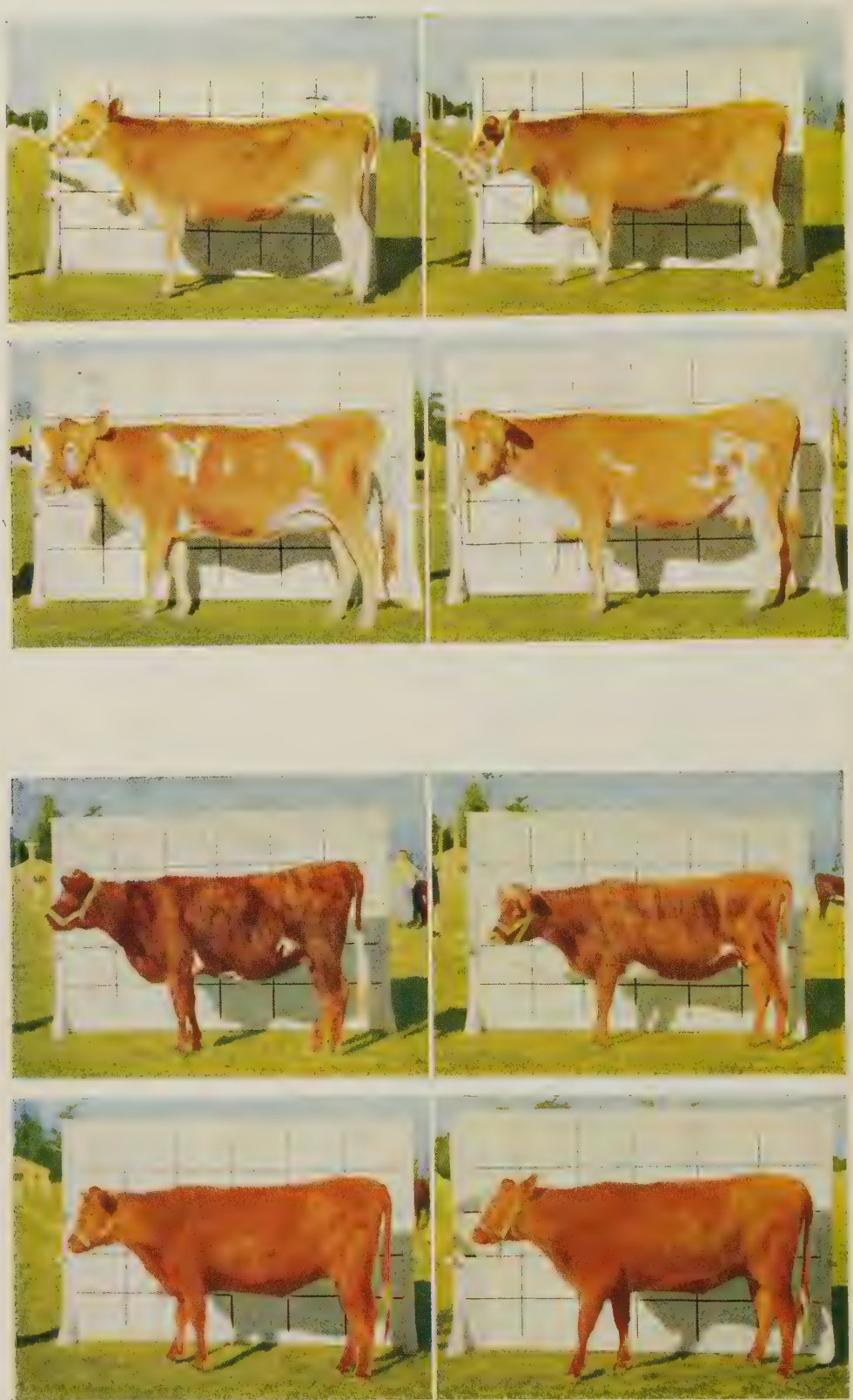


FIG. 21a.—(See text).

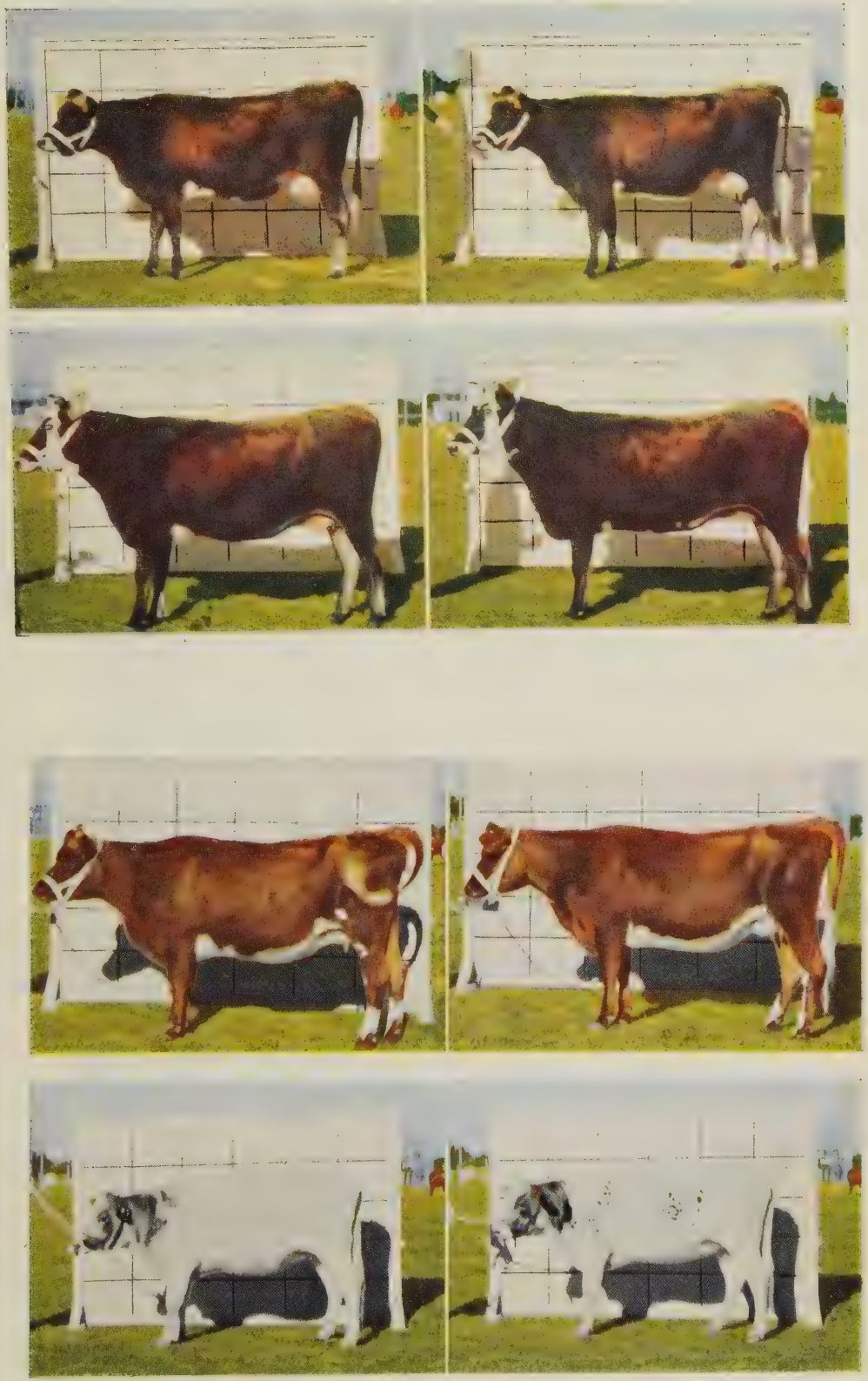


FIG. 21b.—(See text).

FIG. 22.—Five sets of monozygotic bull twins. Note the similarity of colour patterns.

FIG. 23.—The monozygotic twins T35 and T36. Top: left and right side of T35. Bottom: left and right side of T36 (see text).

FIG. 24.—The monozygotic twins T37 and T38. Top: left and right side of T37. Bottom: left and right side of T38 (see text).

nevertheless provide a demonstration of the striking similarities existing within sets of monozygotic twins, and the great range of colour occurring between unrelated animals.

Fig. 22, the photograph of five monozygotic twin bull sets shows that even a half-tone reproduction can give an indication of the within-set similarity and the range of differences which may occur between even a small number of unrelated animals selected at random.

Finally, it must be noted that the colour patterns described in the foregoing are not a reliable criterion of monozygocity if the members of a set of twins have been under the influence of different environments. It has, for instance, been observed in experimental studies at Ruakura that the coat colour of animals kept inside, fades when compared with that of their co-twins kept outside. Lactation is another factor which seems to lighten the coat colour.

It was previously mentioned that even monozygotic twins may show within-set differences with respect to the three characteristics; brindle, roan, and white spotting.

The brindle pattern consists of irregular stripes of black hair on a background of red. Within-set differences of the brindle character cannot easily be measured quantitatively unless very large hair samples are taken. This is due to the irregular shape of the stripes and to the varying position they may occupy in the two members of a set of twins. A small sample from a given anatomical position may give quite an erroneous conception of the extent to which black hair occurs over the whole coat. But although differences of brindle patterns are difficult to measure, they do exist within sets of monozygotic twins as may be judged from the colour reproduction of the twins T43 and T44. This does not, however, mean that the brindle character is of no use as a criterion of monozygocity, as the observed differences within monozygotic twins are relatively small compared with a range commonly occurring in unrelated animals. At one extreme, the brindle pattern in such animals may be expressed by a few thin black stripes on the face, whereas, at the other extreme, the black stripes may be so wide and dense as to give the whole coat an almost jet-black appearance. Even appreciably smaller differences within a set of twins than represented by the extreme would actually be a strong indication of dizygocity, although it has not been possible to formulate a rule indicating the degree of dissimilarities required to make the diagnosis certain.

The roan effect is caused by white hairs interspersed amongst pigmented ones. Because the white hairs, in contrast to the black hairs of the brindle pattern, are not arranged to form a striped pattern, it has been possible to gauge fairly accurately in roan cattle the differences which may exist within presumably monozygotic twins. Hair samples have been taken in the same position from two sets of monozygotic twins which both showed apparent within-set differences in the roan character. The relative numbers of white hairs were determined with the following results :

Per Cent. White Hairs.		Per Cent. White Hairs.
T29 ... ... 12.8		T41 ... ... 27.8
T30 ... ... 25.6		T42 ... ... 35.5

In unrelated animals the roan colour occurs with approximately the same range as the brindle colour. It can be assumed that a set of twins showing extreme differences cannot be monozygotic, and in so far as this is correct the roan character may serve as a useful adjunct in the

diagnosis of monozygocity. However, as in the case of brindle colour, it has not been possible to determine what degree of dissimilarity within a set is critical.

Monozygotic twins may finally show within-set differences in the white spotting pattern. These differences may be both quantitative and qualitative; quantitative in that the relative amount of white varies, and qualitative in that the shape and position of the individual spots vary. The limitations to the usefulness of the white spotting character as an aid in diagnosis of zygocity is due to the circumstances that non-germinal and chance factors influence the expression of the genes which in main determine the extent of the white spots and the type of pattern. As the white spotting pattern cannot be described accurately in objective terms the degree of similarity shown within monozygotic twins can only be appraised by visual comparison. For this purpose photographic reproduction of several sets of monozygotic

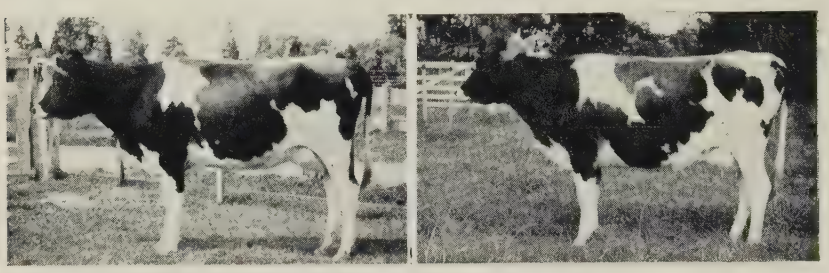


FIG. 25.—The monozygotic twins T53 and T54 showing typical Holstein pattern.

FIG. 26.—The monozygotic twins T49 and T50 showing Holstein pattern.

twins showing white spotting pattern are presented in Figs. 23, 24, 25, 26, 27 and 28. It will be noted that the differences within every one of these sets are of such magnitude that the members could easily be distinguished from each other by this character alone. These differences are, however, relatively small when compared with those occurring between sets which are so great that the animals could be graded on the basis of white spotting into several classes, each so distinct from the others that if two animals of different grades were paired, it would be possible to decide that they could not possibly be monozygotic twins judged by the dissimilarity in white spotting alone. (Compare, for instance, T53 - T54 (Fig. 25) with T49 - T50 (Fig. 26).

The qualitative differences within sets are generally not greater than occur between the two sides of the same animal. This is illustrated in Figs. 23 and 24, which show both sides of two monozygotic

twin sets: T35 - T36, and T37 - T38. Much greater differences are shown between many sets; in fact, several different distinct pattern types can easily be distinguished, each one probably determined by different genetical factors. A set of twins could, therefore, be diagnosed as dizygotic if the members possessed different types of white spotting patterns. In the series of photographs, Figs. 22-27, several pattern types are represented. The Holstein pattern in which the pigmented areas are relatively unbroken and which is also characterized by unpigmented legs is shown by T53 - T54 (Fig. 25). T35 - T36, and T37 - T38 (Figs. 23 and 24), show a typical Ayrshire pattern in which the pigmented areas are broken up into fairly small spots. The "brockle" face pattern characterized by a partly white face as illustrated by T131 - T132 (Fig. 27). T91 - T92 (Fig. 28), show a flecked pattern which is probably a variant of the Ayrshire pattern and which consists of white areas dotted with very small pigmented flecks.

Fig. 27.—The monozygotic twins T131 and T132 showing "brockle face pattern".

Fig. 28.—The monozygotic twins T91 and T92 showing "flecked pattern".

A pattern which is difficult to show by means of photographic reproduction is the "inguinal white," as it is expressed only by a small spot of white on the udder. This pattern is, nevertheless, fairly common and many of the apparently self-coloured sets shown in Figs. 21a and 21b possess this pattern.

Qualitative differences within sets of twins of the magnitude indicated by any two of the above pattern types are seldom encountered, partly, at least, because such sets are not presented for inspection. Within the main patterns, there seem to exist sub-types which, although relatively difficult to separate nevertheless are sufficiently distinct to be useful. Unfortunately, no suitable dizygotic twins were available at Ruakura

to illustrate this point but the two monozygotic sets T35 - T36 and T37 - T38 (Figs. 23 and 24) which incidentally are paternal half sisters, will serve this purpose. The white spottings in both sets are of the Ayrshire type and quantitatively rather similar, but it will be noted that T35 and T36 have several pigmented spots on their tails. They also have spots on their bellies, in which respects they differ from T37 and T38. Such relatively insignificant differences within a set of twins would, therefore, be a strong indication of dizygocity.

(5) *Red cell factors.*

The work of Ferguson *et al.* (12) and Stormont *et al.* (13) on red cell factors in cattle promised to provide a perfect objective test of zygocity of twins. According to these studies, it seemed clear that no two animals ever had identical sets of red cell factors unless they were monozygotic twins. Thanks to the courtesy of Prof. Irwin and Dr. Stormont of Wisconsin, who provided the non-specific sera, Mr. M. Buddle of the Wallaceville Animal Research Station was able to test 22 sets including one set of triplets at Ruakura. Of these, six sets had been diagnosed as dizygotic on preliminary subjective tests. The results from the blood tests shown in Table XI (Appendix II) in every case confirm the subjective test.

It may be noted that three sets of the dizygotic twins differed in one factor each, two sets in two factors and one set in as many as three factors. The differences in blood factor pattern within sets of fraternal twins thus found is somewhat surprising in view of the recent discovery by Owen (14) that most dizygotic twins have identical red cell factors. This has been explained as due to the well-known fact that in bovine twins anastomosis of foetal vessels occur with great frequency, which allows migration of red cells from one foetus to the other. Until the basis for the conflicting results obtained by Buddle and Owen is clarified, an evaluation of the blood factors for the purpose of confirming diagnosis of zygocity of twins must be suspended. Further work on this point at Ruakura awaits an adequate supply of mono-specific sera.

DISCUSSION AND CONCLUSIONS

The ease and certainty by which zygocity can be diagnosed in twins depends primarily on the following three conditions being fulfilled :

(1) There should be a large number of inherited characters, easily measured or otherwise recognizable.

(2) The characters should be influenced to only a small degree by environmental factors; in so far as environment or chance modified the expression of any trait, its usefulness as a diagnostic aid is correspondingly impaired.

(3) There should be a heterogeneous population in respect of the characters employed so as to increase the chances that even such closely related animals as dizygotic twins are relatively unlike.

It is fortunate that cattle exhibit a large number of easily recognizable inherent characters. Of these, the best established are those involving coat colour. Ibsen (8) has enumerated and discussed in detail nineteen separate colour genes occurring in cattle. This list has been revised and extended by White and Ibsen (15), Bogart and Ibsen (16), Bushnell (17), Gilmore *et al.* (18), Deakin (19), Briquet and Lush (20) and Jones (21). Some of these genes occur rarely or not at all in New Zealand dairy cattle, but on the other hand there exists a large number of colour effects which have not yet been fully investigated

although their genetical basis seems certain. The degree to which different colour characters are influenced by environment varies, but environmental factors are on the whole relatively ineffective in causing colour differences within sets of monozygotic twins. Even a colour character such as the white spotting, in which respect monozygotic twins probably never are absolutely identical, is mainly determined by genetical factors (20).

Skin pigmentations depend largely on inherited factors. The mode of inheritance of only one factor has been clearly established: The bs factor which causes black spotting (8). Other characters, such as shades of muzzle colour and the pigmented streaks inside the ears are undoubtedly genetically determined, but as they may represent merely another expression of factors which determines various coat colours, they probably do not increase the number of characters which facilitate the diagnosis of monozygocity at a stage when coat colour is fully developed. In this connection, it may be noted that skin pigmentations, which in an individual are bilaterally symmetrical, are wholly determined by genetical factors, whereas those which show asymmetry are influenced to some extent by prenatal environment and chance.

The number of genetic factors influencing head-shape and body conformation are not known but several factors must be postulated in order to explain all the variations observed amongst individuals even within a single herd. Gowen (22) has shown that the greatest part of the variations in body conformation is due to the genetic control of growth and developmental processes. This finding obtains some support from the following test at Ruakura.

Ten sets of monozygotic twins were selected from a herd of 25 sets to include animals of headsize as similar as possible. The twins were approximately $2\frac{1}{2}$ months old at the time of the test. One member of each set was locked into the calf feeding bails. The remaining animals were then brought in one at a time while the blindfolded author attempted to match the sets by palpation of the heads alone. This was achieved in the case of eight sets. It seems evident, therefore, that differences in head shape are numerous and distinct, and since monozygotic twins are so characteristically similar, a strong genetic basis to conformation is probable.

Table XII has been prepared to show in a general way: (a) the relative number of easily distinguishable phenotypes within each characteristic discussed in this paper; (b) the relative degree to which inherited factors control these characteristics under fairly uniform environmental conditions; (c) the relative facility whereby they can be measured or described in objective terms.

The red cell factor pattern occupies a peculiar position in that it is wholly controlled by genetical factors in single born calves, whereas pre-natal environment partly determines the pattern in twins.

At the present time 75 per cent. of all cows in milk in New Zealand are grade Jersey (24). This stock, however, is rather heterogeneous for two reasons. It has been developed by repeated top-crossing with Jersey sires from a predominantly Shorthorn stock (24), and many farmers make a practice of using bulls of other breeds when their stock has reached some degree of breed purity.

TABLE XII. THE RELATIVE USEFULNESS OF VARIOUS CHARACTERISTICS IN THE DIAGNOSIS OF ZYGOCITY. (see text)

Characteristic.	Relative number of phenotypes.	Relative degree to which genetical factors influence expression.	Relative facility whereby Characters can be measured.
Whorl pattern	++	++	++
Muzzle pattern	+	++	+
Escutcheon pattern	++	++	+
Coat colours :			
(a) white spotting	++	++	++
(b) roan hair	++	++	+++
(c) brindle	++	++	++
(d) others	+++	+++	+
Skin Pigmentation :			
(a) symmetrical	++	+++	++
(b) asymmetrical	+	++	++
Red Cell factor pattern	+++	+++(+)	+++
No. and position of teats	++	+	+++

The heterogeneity of the dairy stock in this country may best be gauged by the fact that over 60 per cent. of the monozygotic twins reared at Ruakura, representing a fair cross section of the present stock, show unmistakable outward signs of breed mixture. The colour reproductions of some of these twins (Figs. 21a and 21b) provided a good index of this situation.

As a final conclusion it is now possible to state that there exists in New Zealand dairy cattle a long series of characters each of which is determined to a considerable degree by genetical factors. The twins classified by the author as monozygotic have proved to be very much alike in all recognizable gene effects shown. This is very strong evidence that they are of monozygotic origin, as it is unlikely that dizygotic twins would show within-set similarities to this degree, considering the number of genes involved and the heterogeneous nature of the stock from which the twins are derived. In all cases the differences frequently observed within monozygotic twins can be attributed to environmental differences, mostly of the type commonly termed pre-natal accidents.

ACKNOWLEDGMENTS

The author wishes to thank Dr. C. P. McMeekan, Superintendent, Ruakura Animal Research Station, for critical reading of the manuscript, and for taking the colour photographs.

REFERENCES

(1) KRONACHER, C. (1932) : *Z. Zuchtung Band, XXV,* Reihe B25, 327-414.

(2) ———— and SANDERS, D. (1936): *Z. Zuchtung Reihe, B.3,* 41-83, 133-220.

(3) HAAK, D. (1942): *Zuchtungskunde, 17,* 326-30.

(4) NEWMAN, HORATIO H., FREEMAN, FRANK N. and HOLZINGER, KARL J. (1937): "Twins : A Study of Heredity and Environment." The University of Chicago Press, Chicago, Illinois, U.S.A.

(5) HANCOCK, John (1949): *N.Z. J. Sci. and Tech., 30* (*Sec. A*), 257-70.

(6) ———— (1949): Unpublished data.

(7) BONNIER, GERT (1946): *Acta Agr. Succana, 1 : 2,* 147-51.
(8) IBSEN, Heman L. (1933): *Genetics, 18,* 441-80.
(9) SHMIDT, J. and KLIESCH, J. (1938): *Kuhn-Arch. 49,* 65 *cit.* Deakin (20).
(10) HABU, Y. (1935): *Bull. Imp. Zootech. Exp. Sta. (Chiba-Shi),* No. 31 *cit.* Deakin (20).
(11) PHILLIPS, Ralph W., SCHOTT, Ralph S. and SPENCER DAMON A. (1946): *J. Heredity 37,* 19-26.
(12) WALLACE, L. (1949): Unpublished data.
(13) FERGUSON, L. C., STORMONT, C. and IRWIN, M. R. (1942): *J. Immunol., 44,* 147-64.
(14) STORMONT, C. and CUMLEY, R. W. (1943): *J. Heredity 34,* 35-41.
(15) OWEN, R. D. (1945): *Science, 102,* 400-1.
(16) WHITE, W. T. and IBSEN HEMAN, L. (1935): *J. Heredity 26,* 75-84.
(17) BOGART, Ralph and IBSEN HEMAN, L. (1937): *J. Genetics, 35,* 31-59.
(18) BUSHNELL, Ralph J. (1940): *J. Heredity, 31,* 253-56.
(19) GILMORE, L. O., PETERSEN, W. E. and FITCH, J. B. (1942): *J. Heredity, 33,* 451-4.
(20) DEAKIN, ALAN (1944): "Identical Cattle Twins and Causes of Spotted Patterns. "Published by the Author.
(21) BRIQUET, R. (Jr.) and LUSH, J. L. (1945): *J. Heredity, 38,* 99-105.
(22) JONES, I. C. (1947): *J. Genetics, 48,* 155-63.
(23) GOWEN, JOHN W. (1933): *Genetics, 18,* 415-40.
(24) HAMILTON, W. M. (1944): The Dairy Industry in New Zealand. *N.Z. Dept. Sci. and Ind. Res. Bull. 89.*

APPENDIX I.

TABLE V. WHORL AND MOUTH PIGMENTATION FORMULAE OF 40 SETS OF MONOZYGOTIC TWINS

No. of Set.	Forehead Whorl.				Poll Whorl.			Back Whorl.		Eye Whorl.		Colour of Mouth.	
	X.	Y.	Type.	Twist.	Left. a n cl	Centre. a n cl	Right. a n cl	cm.	Twist.	Left.	Right.	Tongue Per cent. Pink.	Gum Per cent. Pink.
5	$+\frac{1}{2}$	+1	S	a	+			8	n	+	+	40	20
	0	$+\frac{1}{2}$	N	a	+			8	n	+	+	20	20
8	0	$+\frac{1}{2}$	St Fe	—		+		8	n	—	—	50	50
	0	+1	N	a		+		10	n	—	—	50	60
11	$+\frac{1}{4}$	0	Fo	a	+		+	18	a	—	—	0	30
	$+\frac{1}{4}$	0	Fo	a	+		+	12	a	—	—	0	30
12	$+\frac{1}{2}$	$+1\frac{1}{2}$	N	a				5	a	—	—	100	98
	0	$+1\frac{1}{2}$	N	a				5	a	—	—	100	90
14	$+\frac{1}{4}$	$+1\frac{1}{2}$	S	a	+			15	a	—	—	0	0
	No whorl				+			15	a	—	—	0	0
16	$+\frac{1}{4}$	$-\frac{1}{4}$	Fo	a	+		+	10	n	—	—	100	30
	0	0	Fo	a	+			10	a	—	+	100	20
18			Fe			+		10	a	+	+	0	0
			Fe			+		10	n	+	+	0	0
20	No whorl				+		+	0	a				
								12	n	—	—	0	0
	No whorl				+		+	0	a				
								12	n	—	—	0	0
23	0	+2	S	a		+		0	cl	+	+	0	0
	0	+2	S	a		+		0	a	+	+	0	0
24	0	0	S	a	+			6	n	—	—	100	95
	$+\frac{1}{2}$	0	S	a	+			10	n	—	—	100	95

Table V. Continued.

No. of Set.	Forehead Whorl.				Poll Whorl.						Back Whorl.		Eye Whorl.		Colour of Mouth.	
					Left.			Centre.			Right.											
	X.	Y.	Type.	Twist.	a	n	cl	a	n	cl	a	n	cl	cm.	Twist.	Left.	Right.	Tongue Per cent. Pink.	Gum Per cent. Pink
32	No whorl							+						9	n	—	—	0	0
	No whorl							+						10	n	—	—	0	0
33	0	1½	Fo	cl				+	+					15	a	—	—	0	0
	0	2	Fo	a				+	+					15	a	—	—	0	0
34	0	2	S	cl	+								+	0	a	+	+	0	0
	0	2	S	cl	+								+	0	a	+	+	0	0
37	−1	0	S	cl				+						0	a	—	—	0	0
	−1	0	S	cl				+						0	a	—	—	0	0
38	0	+1	N	a		+								9	n	—	—	30	85
	0	+1	N	cl								+		11	n	—	—	20	85
41	No whorl										+			10	a	+	+	0	0
	No whorl					+					+			10	a	+	+	0	0
47	No whorl					+								0	a				
														7	n	—	—	100	100
	No whorl				+									0	a				
														7	n	—	—	100	100
50	0	−1	Fo	n				+						7	n	—	—	0	0
	0	−1	Fo	a				+						7	n	—	—	0	0
51	0	+½	S	n				+						10	n	—	—	0	0
	+¼	+¼	S	a				+						10	n	—	—	0	0
58	No whorl				+						+			8	a	+	+	40	75
	0	+4		n	+						+			8	a	+	+	30	100

TABLE V. Continued.

No. of Set.	Forehead Whorl.				Poll Whorl.						Back Whorl.		Eye Whorl.		Colour of Mouth.	
					Left.			Centre.			Right.											
	X.	Y.	Type.	Twist.	a	n	cl	a	n	cl	a	n	cl	cm.	Twist.	Left.	Right.	Tongue Per cent. Pink.	Gum Per cent. Pink.
62	0	$+\frac{1}{2}$	Fo	cl				+						9	n	+	+	0	0
	0	$+\frac{1}{2}$	Fo	cl		+					+			9	n	+	+	0	0
63	No whorl				+						+			0	a	+	+	0	0
	No whorl				+								+	0	a	—	—	0	0
69	No whorl				+							+		9	a	+	+	0	30
	No whorl				+								+	9	a	+	+	0	30
70			Fe					+						8	n	—	—	100	30
	+1	$-\frac{1}{2}$	N Fe	a		+					+			10	n	+	—	100	30
72	$+\frac{1}{4}$	−1	Fo	a		+								10	n	—	—	0	0
	0	−1	N	a							+			10	n	—	—	0	0
74	$+\frac{1}{4}$	$+2\frac{1}{2}$	N	a				+						12	a	—	—	100	0
	0	2	N	a	+									12	a	+	—	100	0
75	+1	0	Fo	a					+					11	a	—	+	0	0
	$+\frac{1}{2}$	0	Fo	a					+					11	a	+	—	0	0
76	0	1	S	a	+							+		11	n	+	+	0	0
	$-\frac{1}{2}$	1	S	cl		+					+			11	n	+	+	0	0
83	$-\frac{1}{2}$	0	S	a		+					+			0	a	—	+	0	98
	$+\frac{1}{2}$	0	S	a	+							+		0	a	+	—	0	98
85	No whorl				+									9	a	—	—	0	0
	No whorl				+									10	n	—	—	0	0

Table V. Continued

No of Set.	Forehead Whorl.				Poll Whorl.			Back Whorl.		Eye Whorl.		Colour of Mouth.	
	X.	Y.	Type.	Twist.	Left. a n cl	Centre. a n cl	Right. a n cl	cm.	Twist.	Left.	Right.	Tongue Per cent. Pink.	Gum Per cent. Pink.
87	$+\frac{1}{2}$	$+1\frac{1}{2}$	N	a	+		+	7	a	−	−	0	0
	$+\frac{3}{4}$	$+1\frac{1}{2}$	N	a	+		+	7	a	−	−	0	0
90	$-\frac{1}{2}$	0	S	a	+			0	a	+	+	0	0
	0	$+\frac{1}{2}$	S	n	+		+	0	a	+	+	0	0
91	$+\frac{1}{4}$	3	S	cl		+		8	a	+	+	100	100
	0	2	S	cl		+		8	a	+	+	100	100
93	$-\frac{1}{4}$	+1	S	n		+		0	a	−	−	100	100
	$+\frac{1}{4}$	+1	S	n		+		0	a	−	−	100	100
96	0	0	S	n	+		+	6	a	+	+	100	100
	0	0	S	n	+		+	6	a	+	+	100	100
			Fe										
97	0	$+\frac{1}{2}$	Ce	n	+		+	10	n	+	+	100	99
	0	$+\frac{1}{2}$	Ce	n	+		+	12	n	−	−	100	99
103	0	0	Fo	a			+	18	n	+	+	100	100
	$\frac{1}{4}$	0	Fo	a			+	15	n	+	+	100	100
115	0	$-1\frac{1}{2}$	Fo	n	+	+		5	n	+		0	5
	$+\frac{1}{2}$	−1	Fo	a	+	+		5	n	+	−	0	5
125	0	0	Fo	n		+		0	a	−	+	0	0
	0	0	Fo	a		+		0	a	+	−	0	0
154	0	+2	S	n		+		0	a				
								8	n	+	+	0	99
	0	+2	S	a		+		10	a	+	−	0	99

APPENDIX II.

TABLE XI. CELLULAR ANTIGENS IN CATTLE TWINS AND TRIPLETS

A. Dizygotic.

Set No.	A	B	CE	E	G	H	I	J	K	O	P	Q	R	S	T	TT_2	U	UU_2	V	W	X	XX_2	Y	Z	D′	$E'_{1,2}$	$E'_{1,2,3}$	I′	J′	L′	Z′
113	+	+	+	+		+		+		+								+	+			−	+	+	+			+			−
114	+	+	+	+		+				+								+	+	−		+	+	+	+			+			−
160	+	+	+			+		−	−	+					−		+		+	+				+		−		+			
161	+	+	+	−		+		−		+	−				+	+	+	+	+	+				+		−		+			
76	+	+	+		−	+	+		+	+							−		−	+	−		+		−		+				
77	+	+	+		+				+			−								−	−		+				•				
88	+	+	+	−	+	+		+					+	+				−		+	+	+					+				−
89	+	+	+	−	+	+							+	+						+	+	+					+		−		−
120	+		+															+	+	+	+	+							−		
121	+		+		+			+										+	+	+	—				−				−		
64	+		−		+									−	−						−		+		+		+	+			
93	+				+									-	−						−		+		+		+	+			

B. Monozygotic.

Set No.	A	B	CE	E	G	H	I	J	K	O	P	Q	R	S	T	TT_2	U	UU_2	V	W	X	XX_2	Y	Z	D′	$E'_{1,2}$	$E'_{1,2,3}$	I′	J′	L′	Z′
154	+	+	+	+	+	−			+	+	−			+		−				+	−	+	−	+			+	+		+	−
163	+	+	+	+	+		−		+	+	−			+		−		−		+		+	+	+			+	+		+	−
182	−	+	+	+	+				+	+	−	−		+		−		−		+		+	−	+		−	+	+		+	−
84	+	−	+	+				−		−		−	+		+	+	−		+	+	+	+	+	−	−		−				
85	+		+	+				+					+		+	+	−		+			+	+	+	−		+				
86	+		+			+			−			−		+	+	+		-						+			+				
87	+		+			+		−				−		+	+	+	−		+	+				+			+				

180
186
97
98
106
107
T.17
T.18
T.19
T.20
51
52
C
NC
T.31
T.32
T.41
T.42
T.43
T.44
T.55
T.56
T.83
T.84
SW
SnW

THE BLACKBERRY MITE (*Aceria essigi*)

By AVICE HAMILTON, Entomology Division, Department of Scientific and Industrial Research, Nelson

(*Received for publication 22 October 1948*)

Summary

Aceria essigi, the blackberry mite which causes the "redberry disease" of blackberries, was isolated in the United States in 1921 and is now a recognized pest where commercial blackberries are grown. In 1946 it was found to occur in the Nelson district, New Zealand, and since then has been found in other parts of the country. The mite is recorded from *Rubus* spp. Infested blackberries remain hard and bright red when uninfected berries are ripe and black. A short account of the seasonal cycle is given which appears to apply under New Zealand conditions.

INTRODUCTION

The blackberry mite was found to be injuring blackberries in California in 1921 and since then has been isolated from most of the commercial blackberry-growing areas of the United States. It is believed that the mite originally came from Europe, since it attacks mainly the Himalaya and Evergreen blackberries which are horticultural varieties of European vines.

At first it was considered to be *Eriophyes gracilis* (Essig and Smith, 1922), a species common on the European raspberry, but Hassan (1928) described it as a new species, *E. essigi.* Later Keifer (1941) redescribed the species *E. essigi,* and then in 1944 he divided up the genus *Eriophyes* (Family Eriophyidae) so that the blackberry mite is now known as *Aceria essigi.*

DISTRIBUTION

In the United States, *Aceria essigi* is found damaging blackberries in areas situated along the Pacific coast. It is also recorded in England from the Canterbury district, East Malling, Maidstone and Kirdford. In the autumn of 1946 this mite was isolated from damaged blackberries in the Nelson district, which is the first record of its presence in New Zealand. Since then berries heavily infested with the blackberry mite were found in Whangarei, Tauranga, Blenheim, and throughout the Nelson district as far west as Inangahua. So far no damaged berries have been received from Westland or Taumarunui. The transfer of the blackberry mite from one district to another easily takes place by means of fruit or parts of the blackberry vines.

HOSTS

The Himalaya and Evergreen varieties of blackberry as well as the wild vines are the principal hosts of *A. essigi* in the United States. In England it is recorded from the wild and cultivated blackberries. In New Zealand it is found on the wild blackberry, of which there are several varieties, whose exact distribution is unknown. However, the evergreen variety (*Rubus laciniatus*) is fairly common in both islands.

Hassan (1928) mentions the loganberry and the raspberry as alternative hosts of *A. essigi,* while Massee (1931) also found it on loganberry. Some of the earlier writers record this mite from the dewberry (*Rubus caesus*) and the black raspberry, while Keifer (1944) cites the hosts as *Rubus* spp. and does not refer to any of the previously recorded alternative hosts.

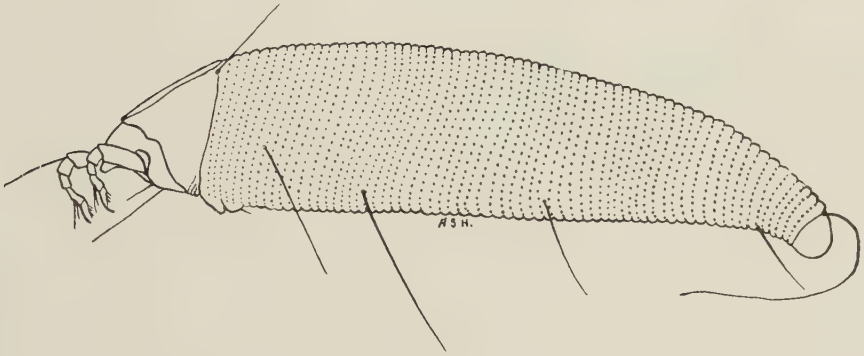


FIG. 1.—The blackberry mite (*Aceria essigi* Hassan), (greatly magnified). (After Hassan.)

NATURE OF INJURY

The blackberries infested with mites do not ripen normally, since the whole berry or perhaps only a few of the drupelets become bright red in colour, hence the name "redberry disease" (Fig. 2). These red berries appear about two weeks before the main crop ripens and at that time are distinguished from the normal fruit by their swollen appearance and bright red colour. When the whole berry is affected it is hard and inedible. The red berries are very conspicuous among the ripe purple fruit, and they often cling to the vine well into the winter. Owing to the extent of the "redberry disease" in the Nelson district, it is difficult to procure ripe blackberries.

FIG. 2.—Blackberries affected by the mite:
(a) Normal fruit and one with single red drupelets;
(b) Typical "red berries".

DESCRIPTION OF MITE

The following description of the Eriophyid mite (*A. essigi*) (Fig. 1) is the one given by Keifer (1941):—

"Female 120-140 μ long, 28-30 μ thick, wormlike, whitish or very light amber. Rostrum 20 μ long, rather small; basal seta present, subapical seta apparently absent. Shield 23 μ long, 24.5 μ wide, median, admedian and a long submedian line present; many short lines and a lateral clear or marked lobe; side granulations; dorsal tubercles 17.5 μ apart, on rear margin; dorsal setae 19.5 μ long, projecting backward. Legs rather short and stocky. Forelegs 23.5 μ long, tibia 3.5 μ long, tibial seta absent, tarsus 5.5 μ long, claw 5.5 μ long, slender, tapering, featherclaw 4 rayed. Hindlegs 20.5 μ long, tibia 3 μ long, tarsus 5.5 μ long, claw 8.5 μ long. Anterior coxae fused with each other and with suboral plate, granular; hind coxae granular. Abdomen with about 65-70 rings, some ventral reduction in ring number; tubercles pointed. Lateral seta 13.5 μ long, on about ring 7; first ventral 44 μ long, on about ring 19; second ventral 30 μ long, on about ring 38; third ventral 12.5 μ long, on about ring 6 from rear; accessory seta present, minute. Female genitalia 16.5 μ wide, 9.5 μ long, coverlap with two concentric, irregular half-rings and basal granules; seta 8 μ long.

Male 105-135 μ long, 25-30 μ thick."

LIFE CYCLE

According to the work carried out on the blackberry mite in England and the United States, the adult mites overwinter under the bud scales of the shoots or any other projected part of the plant. In the spring they migrate to the developing leaves and enter the fruit just after petal fall. The mites congregate at the base of the immature drupelets where they increase rapidly, reaching large numbers (from 100 to 1,400 per fruit) by late summer. In the autumn, when leaving the red berries for hibernation, there is a high mortality among the mites. The life cycle of the mite is probably spanned in one to two weeks; therefore, there will be many overlapping generations during the season.

CONTROL

The blackberry mite may be controlled (Essig and Hoskins, 1944) by a spray of 4 to 8 gallons of lime-sulphur solution or three to four gallons commercial winter oil emulsion per 100 gallons (U.S.) of water. The spray is applied when the leaf buds are just opening during the spring or prior to flowering. Another spray is made up from 5 lb. wettable sulphur applied to the bushes throughout the summer.

ACKNOWLEDGMENTS

The author is indebted to H. H. Keifer, Department of Agriculture, California, for identifying the mite; to Dr. H. H. Allan, formerly Director Botany Division, for information concerning the varieties of blackberries growing in New Zealand; to Mr. R. T. J. Blick, Cawthron Institute, for the photographs; and to Agricultural Instructors, Department of Agriculture, for supplying infested material.

REFERENCES

EDWARDS, W. D., GRAY, K. W., WILCOX, J. and MOTE, D. C. (1935): The Blackberry Mite in Oregon. *Oregon Agr. Exp. Sta. Bull.*, 337.

ESSIG, E. O. and SMITH, E. H. (1922): Two Interesting Blister Mites. *Monthly Bull. Calif. Dept. Agric. 11*, 63.

ESSIG, E. O. (1925): The Blackberry Mite, The Cause of the Redberry Disease of the Himalaya Blackberry and its Control. *California Agr. Exp. Sta. Bull.*, 399.

—— and HOSKINS, W. M. (1944): Insects and other Pests attacking Agricultural Crops. *California Ext. Ser. Cir. 87*, 34.

HANSON, A. J. (1933): The Blackberry Mite and its Control (*Eriophyes essigi* Hassan). *Washington Agr. Exp. Sta. Bull.*, 279.

HASSAN, A. S. (1928): The Biology of the Eriophyidae with Special Reference to *Eriophyes tristriatus* (Nalepa). University of California, *Pub. in Ent., 4*, 380.

KEIFER, H. H. (1941): Eriophyid Studies XI. *California Dept. Agr. Bull., 30*, 205.

—— (1944): Eriophyid Studies XIV. *Aceria* Keifer, new genus. *California Dept. Agr. Bull., 33*, 22

—— (1946): A Review of North American Economic Eriophyid Mites. *J. Econ. Ent., 39*, 563-70.

MASSEE, A. M. (1931): The Blackberry Mites. *J. Pom. Hort. Sci., 9*, 298-302.

A RECENT OUTBREAK OF HONEY POISONING

PART VII.* OBSERVATIONS ON THE TOXICITY AND TOXIN OF THE TUTU (*Coriaria Arborea* Lindsay)

By T. Palmer-Jones, Animal Research Station, Wallaceville, and E. P. White, Ruakura Animal Research Station, Hamilton

(*Received for publication 5 August 1948*)

Summary

Samples from various parts of the plant collected in different localities were examined for toxins other than tutin, using a combination of chemical separations and toxicity tests on guinea-pigs.

There is no evidence for significant amounts of mellitoxin or other toxins, particularly those of poisonous honey in the tutu plant. Tutin was the only toxin isolated from young shoots, leaves, and stems, of tutu. In several cases about 65 per cent of the tutin estimated by toxicity measurements was isolated.

Honey-dew washed off one sample gave mellitoxin and evidence for other toxins, but only a trace of tutin, indicating that tutin is converted to the other toxins on passage through the hopper *Scolypopa australis*.

Observations were made on the toxicity of different parts of the plant; the very young shoots are the most toxic part.

The efficiency of different methods of extracting the total toxins of the plant was investigated.

Quercetin was identified in the plant.

Introduction

In previous papers of this series it was shown that about 20 per cent of the total toxic effect of poisonous honey is due to mellitoxin, $C_{15}H_{18}O_7$, which is closely allied to tutin. Evidence was presented that the toxin in the honey originates from honey-dew produced on tutu (*Coriaria arborea*† Lindsay) leaves and stems by the passion-vine hopper and that this honey-dew, in which mellitoxin was identified (1), is collected by bees.

It will be seen that previous work on the occurrence of toxic honey, while indicating the source of the poison and the mechanism underlying its production, left unanswered several questions concerning the role of tutu. Thus it was necessary to determine whether mellitoxin or other honey toxins occur in *C. arborea* and are transmitted mechanically by the hopper, or whether they appear solely as a result of changes in tutin during its passage through the hopper. It was necessary further

---

*Continued from *N.Z. J. Sci. and Tech.*, *29* (*Sec. A*), 107-43 (1947).

† *C. arborea* Lindsay is the plant reported by Cheeseman, "Manual of the New Zealand Flora," 2nd Ed. Wellington, 1925, pages 546-7 as *C. ruscifolia* Linn. and reported under this name by previous investigators.

to determine whether tutin is present in honey-dew or toxic honey, i.e. whether any of the toxic principle of *C. arborea* passes unchanged through the hopper.

In searching for mellitoxin and other toxins it was necessary to be certain of the efficiency and completeness of the chemical methods adopted. For this reason it was essential to check chemical extractions with biological toxicity measurements. In the absence of these biological checks we could not accept as final the work of previous investigators (2) who found only tutin present. The plan of the work therefore was to determine the toxicity of different parts of *C. arborea* by using the guinea-pig, to carry out chemical extractions of which the completeness was measured by further toxicity tests, and then to search the extracts for mellitoxin or other toxins. Similar methods of chemical extraction with biological control were used in an examination of the honey-dew. Throughout the work chemical procedures known to extract a high proportion of the toxicity of honey were used (e.g. ethyl and amyl acetate extractions).

Experimental

The Toxicity of Different Parts of Coriaria arborea.

The guinea-pig was used in toxicity tests because of its susceptibility to tutin and mellitoxin (3). The animals used weighed approximately 400 g. and the material under test was finely ground, suspended in water, and administered by stomach tube (4).

In determining the toxicity of parts of *C. arborea,* particular attention was paid to young shoots, as previous workers agreed that they were highly toxic (see Table I).

Table I.

Toxicity to Guinea Pigs of Young Shoots of Tutu

Source of Dried and Ground Shoots and Date of Collection.	Karapiro Sep. 1947.	Pongakawa Oct. 1947.	Pongakawa Nov. 1947..	Akatarawa Nov. 1947.	Nelson Jan. 1948.
Approximate lethal dose for 400g. guinea-pigs.	0·3g.	0·1g.	0·4g.	0·2g.	0·8g.
Remarks.	Shoots in young stage up to two feet in length.	Shoots in very young stage.	Shoots fairly well developed.	Shoots mostly in very young stage.	Shoots not well developed.

The dry weight of the shoots was less than one-third that of freshly picked material.

As the shoots mature there is apparently a reduction in toxicity, in agreement with lower yields of tutin obtained by Easterfield and Aston

TABLE II. TOXICITY TO GUINEA PIGS OF LEAVES, STEMS AND ROOT PARTS OF TUTU

Nature of Dried and Ground Plant Material.	Mature Leaves.			Young Leaves.	Stems.			Root Cortex.		Root Core.	
Source and Date of Collection.	Kara-piro Sep. 1947.	Ponga-kawa Nov. 1947.	Pine-haven Nov. 1947.	Ponga-kawa Nov. 1947.	Kara-piro Sep. 1947.	Ponga-kawa Nov. 1947.	Pine-haven Nov. 1947.	Kara-piro Sep. 1947.	Ponga-kawa Nov. 1947.	Kara-piro Sep. 1947.	Ponga-kawa Nov. 1947.
Approximate lethal dose for 400g. Guinea-pigs	0·6g.	0·5g.	1·4g.	0·3g.	0·5g. causes no symptoms			0·25g. causes hypersensitivity only		0·6g. causes no symptoms	
Remarks					Maximum dose. Stems up to ¼ in. thick			Maximum dose		Maximum dose	

(2) from shoots collected later in the season. Detailed toxicity tests on one sample of shoots indicated that the toxicity was equally distributed throughout the material.

Results of toxicity tests on other plant parts of *C. arborea* are given in Table II.

Great difficulty was experienced in dosing certain of these plant parts, particularly the root core. Table II indicates that material from the stems, root cortex, and root core, has a low toxicity. It will be noted that young leaves are much more toxic than mature ones.

Table III presents results obtained from toxicity tests on the fruit and associated plant parts of *C. arborea* collected at Pongakawa.

Table III.

Toxicity to Guinea Pigs of Berries, Raches and Racemes of Tutu

Dried and Ground Plant Part.	Toxicity tests using 400g. guinea-pigs.
Ripe berries	1·4g. no symptoms
Unripe berries	0·2g. lethal dose
Rachis etc.—ripe fruit removed	2·7-3·0g. lethal dose
Rachis etc.—unripe fruit removed	1·3g. lethal dose
Flowering Racemes	0·4g. lethal dose

The ripe fruit and associated raceme parts are relatively non-toxic, while from ripe fruit only a trace of tutin could be isolated which is in line with the findings of Easterfield and Aston (2), (5). Green fruit and associated raceme parts, also flowering racemes, are highly toxic. The appearance of much toxin in green seeds and flowers, and its disappearance in ripe seeds, is common in plants, occurring for example in hemlock and in several leguminous plants, where the toxic principles are alkaloids.

The lethal dose of unripe tutu berries (dried) for pigeons was found to be 0.6 g., while that of young shoots (dried) collected at Pongakawa in October 1947 was 0.3 g., being in each case three times the lethal dose required for guinea-pigs. Dosing was carried out by injecting an aqueous suspension of the plant material into the birds' crops by means of a rubber catheter attached to a hypodermic syringe. It is of interest to note that the lethal dose of picrotoxin, administered similarly to pigeons, was found to be 11 mg. per kg., and that of mellitoxin 30 mg. per kg. Fitchett (6) estimates the lethal dose of tutin for pigeons as slightly over 10 mg. per kg. These birds are thus very much more resistant to mellitoxin than to picrotoxin and tutin. They would be of less use in testing plant parts for traces of tutin than the more susceptible guinea-pig.

A dose of about 1 mg. of tutin produced nausea, vomiting, and incapacity for work extending over 24 hours in a healthy, full-grown man (2). Humans are much more susceptible on a body-weight basis to tutin than rats and guinea-pigs. The lethal dose of tutin for a 400 g.

guinea-pig is 0.5 mg. (3), and it appears probable that 5 mg. would be fatal to a human. The lethal dose of young shoots for guinea-pigs commonly varies from 0.1 to 0.4 g., and that of ripe fruit is about 2 g. (both on a dry weight basis); it would seem that these doses correspond to 3-12 g. and 40 g. respectively on a wet weight basis in the case of man.

CHEMICAL EXTRACTIONS

Chemical extractions were checked throughout by toxicity tests on guinea-pigs. The toxicity was determined on the original material as described in the first section, and similar methods were employed for determining the toxicity of the extract and residue.

The biological method had a further advantage in that it provided a relatively rapid and simple means of following the course of chemical extraction. Only those fractions showing significant toxicity were subjected to the tedious chemical methods of isolating and identifying the active toxins present. Furthermore, the unknown chemicals which account for much of the toxic effect of poisonous honey and honey-dew, if present, could only be detected by their toxicity.

Extractions of Plant Parts to Check the Presence of Mellitoxin and Toxins other than Tutin

The first step consisted in testing the efficiency of removal of toxins from plant parts by various solvents.

Young Shoots:

In several cases small amounts (e.g. 30 g.) of dried and ground plant material were refluxed with much water (400-600 ml.) for two hours. The first extract contained 33-80 per cent of the estimated toxin. Repetition of the process gave an extract containing a further few per cent of the toxin, and a third extraction removed little if any toxin. Residual plant material was usually non-toxic in high doses. In three instances the water extraction removed over 75 per cent of the toxicity; in two instances only 30-50 per cent. Small-scale Soxhlet extraction using water gave in one case an extract containing 78 per cent of the toxin.

Twenty grams of shoots were refluxed with five lots of 200 ml. of amyl acetate, each period being one hour. The combined extracts were concentrated *in vacuo*, and taken up in water, giving an extract containing 63 per cent of the toxin. Twenty grams of shoots refluxed for three hours with 400 ml. of chloroform gave an extract containing only 25 per cent of the toxin.

Small-scale Soxhlet extractions (1½ days) of various samples gave, after evaporation of solvent and taking-up in water, extracts containing 41-91 per cent toxin—amyl acetate 53 per cent, 74 per cent; alcohol 78 per cent; chloroform 41 per cent; ethyl acetate 91 per cent.

Leaves:

An amyl acetate Soxhlet extract contained 100 per cent of the toxin.

Root Cortex:

It has been found that 0.25 g. causes hypersensitivity.

Water extracts prepared by refluxing with large volumes of solvent, or by Soxhlet extraction, contained little, if any, toxin, and the recovered cortex still caused hypersensitivity in 0.25 g. doses. Small-scale Soxhlet extracts (eight hours) using alcohol or amyl acetate gave aqueous

solutions with lethal doses equivalent to about 0.9 g. A few milligrams of colourless crystals which separated in the preparation of the aqueous solution were non-toxic.

Extraction of Toxins from Aqueous Concentrates by Solvents, and Removal of Organic Acids

In several cases aqueous concentrates to which salt had been added were extracted with six lots of ether (equal volumes). The extracts usually contained about 75 per cent of the toxin, and the aqueous phase the remainder, but in some cases (including one using a continuous extractor) extraction was not so effective. Ethyl acetate also removed most of the toxin in a few extractions. Concentrates were normally treated with sufficient sodium bicarbonate to neutralize the large amount of organic acids in the plant (7). Ether extraction (six lots) of these solutions, left, in several cases, only 2-6 per cent of the total toxin in the acid fraction.

Examination of Tutu Samples for Tutin and Other Toxins Young Shoots collected at Karapiro, September 1947:*

It has been found that 0.3 g. of shoots is the lethal dose by stomach tube, corresponding to 0.16 per cent tutin.

Some 2.24 kg. was extracted in 300 g. lots continuously with ethyl acetate, the extracts batched, and most of the solvent removed; water was added and the ethyl acetate distilled off. Black tar which separated was removed. The lethal dose of the clear extract was equivalent to about 0.2 g. of shoots (see Table IV).

Excess salt was added (retaining the precipitate) and the whole extracted with six lots of ether (equal volumes), giving an ether extract (A). The aqueous phase, freed from ether by vacuum concentration retained about 25 per cent of the original toxicity. It was extracted with five lots of ethyl acetate (equal volumes) giving an ethyl acetate extract (B). The aqueous phase, freed from ethyl acetate was non-toxic. Concentration of this solution gave crystalline material also non-toxic.

The ether extract (A) was concentrated, water added, and vacuum concentrated. A yellow precipitate which appeared was not filtered off. Sodium bicarbonate was added until the solution was just alkaline, when it was re-extracted with six equal volumes of ether. The bicarbonate solution of the organic acids contained only about two per cent of the total toxicity. The new ether extract was concentrated and taken up in water. A yellow crystalline material sparingly soluble even in hot water separated. Concentration to 200 ml. gave further yellow material. This was combined with a similar substance which had separated in the ether phase during the extractions, giving material (C).

Further concentration of the solution (removing some tarry substance and clarifying with charcoal) gave crystalline tutin, totalling 1.3 g. The mother-liquor was extracted with ether, and a further 0.47 g. of crystalline tutin recovered. This tutin (and 0.3 g. from other sources) was recrystallized from hot water (charcoal) giving 1.35 g. of clean crystals (m.p. 206°c.), and 0.60 g. less pure. This tutin showed a lethal dose of 1.2 mg. per kg. The melting point of pure tutin is reported as 209-210°c. (7), 208-209°c. (2), (5), and 211-213°c. (7).

* All values in this work are in terms of air-dry weight of the plant.

The ethyl acetate extract (B) was concentrated and taken up in water giving a thick syrup which gave no precipitate on standing. If much mellitoxin were present its separation at this stage would be expected. The syrup was diluted with water, and extracted with three lots of ether, giving an extract (B1). The aqueous phase was extracted with five lots of double its volume of amyl acetate giving an extract (B2). The remaining aqueous phase was non-toxic.

The extract (B1), treated similarly to the main ether extract (A), gave the yellow material, and on further concentration, 180 mg. of tutin, m.p. 185°C., with the correct lethal dose for tutin. The mother-liquor, concentrated and taken up in acetone, was passed through an alumina column, when a further 70 mg. of tutin with the correct lethal dose was obtained.

The extract (B2) was dried with sodium sulphate, evaporated, and taken up in water. Concentration to 20 ml. gave yellow crystalline material. As further concentration did not give clear crystals, concentration was continued and the residue taken up in acetone and passed through an alumina column when most of the coloured material was retained. The eluted fraction gave on concentration in aqueous solution 0.35 g. of crystals, m.p. 193°C., showing no further depression of melting point on admixture with tutin and having the expected lethal dose. The crude tutin isolated amounted to 2.30 g., corresponding to 64 per cent of the theoretical yield.

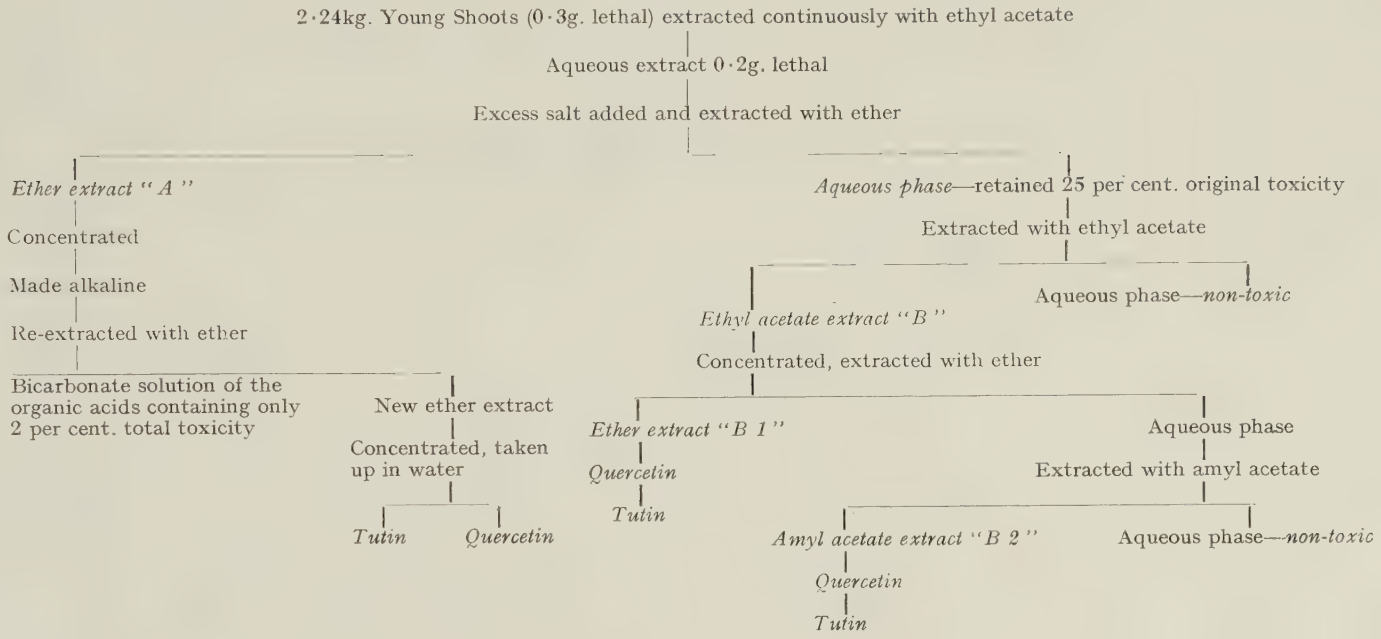
The yellow material (C) was purified by repeated solution in acetone and precipitation with water, forming fine cream-yellow needles, 0.46 g., m.p. 312°C. (dec.). The properties of this material agreed with those of quercetin, m.p. 305-317°C. (8), and 312-316°C. in other recent literature. For analysis it was dried at 120°C. and 15 mm. for three hours. (Found: water loss 10.5, 10.6. $C_{15}H_{10}O_7$, $2H_2O$ the reported dihydrate of quercetin requires water loss corresponding to $2H_2O$, 10.6.) (Found: C, 59.1; H, 3.1. $C_{15}H_{10}O_7$ requires C, 59.60; H, 3.31.) Refluxing with acetic anhydride and sodium acetate gave colourless needles, recrystallized from hot alcohol, in which they are sparingly soluble, m.p. 191°C. (reported for penta-acetyl quercetin, m.p. 191°C (5), 193°C. (9), and up to 200°C. (cor.) in other recent literature (10)). For analysis it was dried at 130°C. and 15 mm. for four hours. (Found: C, 58.7, 58.2; H, 4.2, 4.5. $C_{15}H_5O_7(CH_3CO)_5$ requires C, 58.59; H, 3.90). Quercetin fed in doses up to 273 mg. was non-toxic.

Leaves Collected at Karapiro, September 1947:

It has been found that 0.6 g. is the lethal dose by stomach tube, corresponding to 0.08 per cent tutin.

Treatment of 1.47 kg. as for shoots gave an extract whose lethal dose corresponded to 0.65 g. of leaves. The ether extract contained nearly all the toxin, and the subsequent ethyl acetate extract and final aqueous residues contained no more than a few per cent of the total toxin. From the ether extract much non-toxic, quercetin-like material was obtained, and 0.47 g. of tutin, m.p. 196°C., purified by chromatography to a m.p. 206°C. $[\alpha]_D + 7.6°$ (c = 1.315 in ethanol), reported for tutin $[\alpha]_D^{19.5°} + 9.25°$ (c = 2.5 in ethanol) (2, 5). (Found: C, 61.0; H, 5.8. $C_{15}H_{18}O_6$ requires C, 61.2; H, 6.1).

Table IV. Diagram Showing Extraction of Young Shoots Collected at Karapiro, September, 1947



Acetylation of tutin with acetic anhydride and pyridine as described in (7), gave acetyl tutin, recrystallized from methanol-water as needles, m.p. 178°c., sintering from 176°c., reported 176°c. (7). (Found: C, 60.4, 60.5; H, 5.3, 5.8. $C_{17}H_{20}O_7$ requires C, 60.7; H, 5.95).

The ethyl acetate extract gave 0.45 g. of quercetin-like material, non-toxic, and its mother-liquor a few milligrams of tutin. The final aqueous solution on concentration gave non-toxic crystalline material. The crude tutin isolated amounted to 0.47 g. corresponding to 40 per cent of the theoretical yield.

Leaves and Stems collected at Rotoma, February 1948:

Honey-dew was removed from this sample by previous washing with water. (1.3 g. is the lethal dose, corresponding to 0.03 per cent tutin.) Treatment of 3.17 kg. as for shoots gave an ethyl acetate extract in water, the lethal dose of which corresponded to 1.2 g. of plant material. The ether extract gave 0.6 g. of quercetin-like material and the aqueous solution had a lethal dose corresponding to 2.5 g. plant material. 0.46 g. of tutin, m.p. 200°c., was obtained using chromatography. Subsequent ethyl acetate extraction removed about 10 per cent of the total toxin. The aqueous solution of this extract was extracted with amyl acetate, the new extract giving 0.34 g. of impure quercetin, and on chromatographing, 30 mg. of tutin, m.p. 202°c.; there was no evidence for mellitoxin or other similar materials. The crude tutin isolated amounted to 0.49 g., corresponding to 51 per cent of the theoretical yield.

Ripe Fruit collected at Rotoma, February 1948:

Up to 1.4 g. dose had no effect. A quantity (178 g.) was extracted as above giving an extract in water, the lethal dose of which corresponded to 2.1 g. of fruit. The ether extract gave almost the same lethal dose, and subsequent ethyl acetate extracts and aqueous residues were non-toxic in large doses. The ether extract gave an oily residue containing crystals of tutin, and on chromatography, 5 mg. of tutin, m.p. 207°c., showing no melting point depression on admixture with tutin from shoots, were obtained. No evidence for other toxins was obtained from the ether or subsequent fractions, and no crystals of tutin could be recognized in the ethyl acetate extract.

Examination of Honey-dew Removed from Leaves and Stems Collected at Rotoma to Check for Tutin

Some 51.5 lb. wet-weight (16.7 lb. dry-weight) of leaves and stems were collected at Rotoma on 7th February 1948. This material was heavily infested with *Scolypopa australis,* which was actively excreting honey-dew. The honey-dew was washed off the plant material with large volumes of cold water. The water was removed by distillation at 15 mm., giving 245 g. of a thick dark syrup, $[\alpha]_D^{23°}$ + 52° (c = 1.34 in water), pH = 4.2. The lethal dose was 0.9 g.

A quantity of honey-dew (210 g.) in 150 ml. of water was extracted five times by the amyl acetate method described under (C), when 37 per cent of the total toxin was extracted. The aqueous solution of the amyl acetate extract was then extracted with two lots of petroleum ether to remove wax, and with five lots of ethyl acetate, and the extract evaporated and taken up in water. This solution was extracted with three equal volumes of chloroform, and the extract concentrated and taken up in water.

The chloroform extract was of low toxicity. Using chromatography and concentration of aqueous solutions, a few milligrams of crystals similar in appearance to tutin were obtained. These had a micro melting point of 206 to 208°C., identical with that of tutin under the same conditions; the mixed melting point was also the same. The aqueous phase after chloroform extraction contained nearly all the toxicity of the original amyl acetate extract. It was concentrated to a small bulk, when 26 mg. of mellitoxin, m.p. 248°C. (dec.), sintering from 245°C., was obtained.

Subsequent ether extracts and aqueous residues contained little toxin.

The honey-dew remaining after the amyl acetate extraction was non-toxic, indicating destruction of 67 per cent of the total toxicity; this does not occur in similar extractions of poisonous honey.

The Effect of Incubation on Tutin in Honey, and Its Recovery

This experiment was designed to determine whether tutin underwent any chemical change on prolonged incubation in honey, and to give information on the recovery of tutin by the chemical methods used in extracting toxins of honey and honey-dew. It has been found that 50 to 80 per cent of the total toxicity of poisonous honey can be extracted by refluxing with five lots of amyl acetate.

Tutin (144 mg.) was mixed with 203 g. of non-toxic honey similar in nature to toxic Pongakawa honey, and kept at 37°C. for four weeks. An equal volume of water was added, and the solution was refluxed with five separate lots of amyl acetate for one hour each, on a hot-plate, with continuous stirring. The extracts were united, most of the solvent removed *in vacuo,* water added, and the remaining solvent removed *in vacuo.* Toxicity tests on the aqueous solution before and after this extraction showed that toxicity was accounted for quantitatively by the tutin added. The aqueous extract was then neutralized with sodium bicarbonate and extracted with six equal volumes of ethyl acetate (this solvent would extract any mellitoxin). The solvent was removed *in vacuo* and the residue taken up in water. The solution was extracted with two lots of petrol-ether, then with three equal volumes of chloroform.

The chloroform extract contained by toxicity measurement 20 per cent of the original tutin, and from it was isolated by chromatography 23 mg. of tutin. The aqueous mother-liquor was concentrated and allowed to stand, when no mellitoxin separated out, and the only crystals apparent on further concentration appeared to be tutin. Water was added, and the solution extracted with six lots of ether. From the ether extract, 21 mg. of tutin, m.p. 206°C., was isolated, and a further 18 mg. of less pure tutin was isolated by chromatography of the mother-liquors. The aqueous solution after ether extraction had very little toxicity. Allowing for portions of extracts used for toxicity tests, approximately 100 mg. of tutin should have been isolated, whereas a total of 62 mg. was obtained.

DISCUSSION

The only toxin reported in various *Coriaria* species by several investigators is tutin, $C_{15}H_{18}O_6$. Further work by the present authors on poisonous honey and honey-dew has established only traces of tutin, the mellitoxin reported by Sutherland (1), and the bulk of the toxin which still remains unknown.

No evidence for the presence of mellitoxin and other honey toxins has been obtained in tutu, as a result of exhaustive examination of several samples of tutu including young shoots, leaves, and a sample of leaves and stems from which honey-dew was removed. A sparingly soluble pigment occurring in most fractions containing tutin was identified as quercetin, previously reported in *C. myrtifolia* (11). Quercetin or an isomer was found in *C. thymifolia* (2, 5). The amount of tutin isolated was in several cases about 65 per cent of that calculated from toxicity estimations. Allowing for slight inaccuracies in toxicity estimation based on the guinea-pig, and the difficulty of isolating small amounts of tutin, it is considered that tutin is the only significant toxin in the plant.

A sample of honey-dew extracted by the normal methods gave a trace of tutin, a considerable amount of mellitoxin, and evidence of other honey toxins. Tutin was not changed by prolonged incubation at 37°C. in honey, and reasonable recovery of tutin by the method used for investigating honey-dew was obtained.

It is evident from these findings that in passing through the hopper (*Scolypopa australis*), tutin is converted almost entirely to mellitoxin, and presumably other honey toxins, a fact of great biological interest.

The plant parts from which honey-dew was removed at Rotoma were of relatively low toxicity, 0.03 per cent tutin compared with 0.1 to 0.5 per cent in most young shoots. Hoppers will produce honey-dew from young shoots to the same extent as from other parts of the tutu and it is indeed fortunate that the shoots are not abundant at that period of the year when conditions favour maximum production of toxic honey-dew.

Continuous extraction of the plant with ethyl acetate appeared to give the best extraction of tutin, and was used in large-scale work; extractions with water did not give consistently satisfactory results. Complete removal of tutin is effected in the aqueous phase by a few shakings with ethyl acetate. Organic acids were removed by addition of sodium bicarbonate (7), and extraction with ether, when only a trace of tutin remained in the acid fraction.

ACKNOWLEDGMENTS

Thanks are due to Mr. C. R. Paterson, Apiary Instructor, Horticulture Division, Department of Agriculture, Hamilton, for collecting some of the samples used; to Miss M. Taylor for help in dosing guinea-pigs; and to Mr. N. M. Lauriston for assistance in extraction work.

REFERENCES

(1) SUTHERLAND, M. D. and PALMER-JONES, T. (1947): *N.Z. J. Sci. and Tech., 29* (*Sec. A*), 129-33.
(2) EASTERFIELD, T. H. and ASTON, B. C. (1901): *J. Chem. Soc., 79,* 120-6.
(3) PALMER-JONES, T. (1947): *N.Z. J. Sci. and Tech., 29* (*Sec. A*), 121-5.
(4) ——— (1947): *N.Z. J. Sci. and Tech., 29* (*Sec. A*), 107-14.
(5) EASTERFIELD, T. H. and ASTON, B. C. (1900): *Trans. N.Z. Inst., 33,* 345-55.
(6) FITCHETT, F. (1908): *Trans. N.Z. Inst., 41,* 286-366.
(7) SLATER, S. N. (1943): *J. Chem. Soc.,* 50-1.
(8) RUPE, H. and SCHAERER, M. (1932): Klein's "Handbuch der Pflanzen Analyse," Vol. 3, 2nd Part, Vienna, p. 876.
(9) KARRER, P. and SCHWARZ, K. (1928): *Helv. Chim. Acta, 11,* 916-9.
(10) GUTHRIE, J. D., O'CONNOR, R. T., STANSBURY, M. F. and SAVICH, T. R. (1944): *J. Am. Chem. Soc., 66,* 1794-5.
(11) PERKIN, A. G. (1900): *J. Chem. Soc., 77,* 428-32.

BULLETINS OF THE DEPARTMENT OF SCIENTIFIC AND INDUSTRIAL RESEARCH

No.

1-4. (Out of print.)
5. PLANT-BREEDING METHODS AND RESULTS. F. W. Hilgendorf. Price, 6d.
6. REPORT ON THE FISCHER PROCESS. H. O. Askew. Price, 6d.
7. NEW ZEALAND WOOL FIBRES. (Out of print.) E. F. Northcroft. Price, 6d.
8. SUMMARY OF INVESTIGATIONS ON NEW ZEALAND COAL. W. Donovan. Price, 6d.
9. HIGH- AND LOW-TESTING MILK FOR CHEESEMAKING IN NEW ZEALAND. (Out of print.) P O Veale.
10. LOW-TEMPERATURE CARBONIZATION OF BLENDED NEW ZEALAND COALS. (Out of print.) W. G. Hughson.
11. BUILDING STONES OF NEW ZEALAND. P. Marshall. Price, 4s.
12. REPORT OF THE NUTRITIVE VALUE OF MEAT MEALS. (Out of print.) E. A. Pope.
13. YIELD OF CHEESE PER POUND OF BUTTERFAT. P. O. Veale. Price, 9d.
14. LOW TEMPERATURE CARBONIZATION OF NEW ZEALAND COALS. 1. WAIKATO COAL. W A. Joiner. Price, 9d.
15. PEACH CULTURE. (Out of print.) K. M. Curtis and T. Rigg. Price, 1s.
16. COLD STORAGE OF FRUIT INVESTIGATIONS OF 1927 AND 1928. L. W. Tiller. Price, 6d.
17. PIG RECORDING, BACON-MANUFACTURE, AND THE PIG INDUSTRY IN NEW ZEALAND. M. J Scott, C. R. Barnicoat, and F. R. Callaghan. Price, 1s. 6d.
18. GOITRE IN SCHOOL-CHILDREN. (Out of print.) R. A. Shore and R. L. Andrew.
19. MOUTERE HILLS PASTURES. T. Rigg and H. O. Askew. Price, 9d.
20. LOW TEMPERATURE CARBONIZATION OF NEW ZEALAND COALS. 2. KAITANGATA AND LINTON COALS. W. A. Joiner. Price, 6d.
21. STANDARDIZED CHEESE AND CHEESE ANAYLSIS. (Out of print.) F. H. McDowall.
22. PRELIMINARY HOST-LIST OF THE ENTOMOPHAGOUS INSECTS IN NEW ZEALAND. (Out of print.) E. S. Gourlay.
23. RELATION OF STORAGE TEMPERATURE TO THE OVERSEAS CARRIAGE OF SOME VARIETIES OF NEW ZEALAND EXPORT APPLES. L. W. Tiller. Price, 6d.
24. A CLASSIFICATION OF THE WAIKATO TYPE OF COAL. W. H. Penseler. Price, 1s. 9d
25. BRONZE BEETLE RESEARCH. A. M. Lysaght. Price, 1s. 6d.
26. INFLUENCE OF SEASON AND FERTILIZER ON THE YIELD AND COMPOSITION OF TYPICAL DAIRYING PASTURES. T. Rigg. Price, 2s.
27. TREATMENT AND DISPOSAL OF DAIRY WASTE WATERS. Out of print.)
28. WHEAT RESEARCH INSTITUTE : FIRST ANNUAL REPORT, 1929-30.
29. LOW TEMPERATURE CARBONIZATION OF NEW ZEALAND COALS. 3. MILLERTON AND BLACK-BALL COALS. W. A. Joiner. Price, 1s.
30. WOOL RESEARCH.
31. INVESTIGATIONS INTO PASTURE PRODUCTION. A. W. Hudson. Parts I, II, III issued. Price, 5s.
32. BUSH SICKNESS. H. O. Askew, T. Rigg, L. I. Grange, N. H. Taylor, and others. Price, 2s. 9d.
33. BUTTERFAT LOSSES IN BUTTERMILK. W. H. Udy. Price, 1s.
34. NUTRITIVE VALUE AND VITAMIN EFFICIENCY OF NEW ZEALAND DAIRY PRODUCE.
35. WHEAT RESEARCH INSTITUTE : SECOND ANNUAL REPORT, 1930-31. F. W. Hilgendorf. Price, 1s. 6d.
36. OVERRUN OF NEW ZEALAND BUTTER. (Out of print.) G. M. Valentine.
37. THE EFFECT OF SALT ON THE QUALITY OF CHEDDER CHEESE. (Out of print.) W. Riddet, G. M. Valentine, F. H. McDowall, and L. A. Whelan.
38. FACTORS AFFECTING THE RATE OF RIPENING OF NEW ZEALAND EXPORT CHEESE. (Out of print.) W. Riddet, G. M. Valentine, and F. H. McDowall.
39. BRIQUETTING OF NEW ZEALAND COALS. W. A. Joiner, W. G. Hughson, and A. K. R. McDowell. Price, 9d.
40. GOLD-BEARING CONGLOMERATES OF CENTRAL OTAGO. E. O. Macpherson. Price, 9d.
41. THE RELATION OF STORAGE TEMPERATURE TO THE OVERSEAS CARRIAGE OF SOME FURTHER VARIETIES OF NEW ZEALAND EXPORT APPLES. (Out of print.) L. W. Tiller.
42. WHEAT RESEARCH INSTITUTE : THIRD ANNUAL REPORT, 1932-33. (Out of print.)
43. HAWKE'S BAY EARTHQUAKE. (Out of print.) F. R. Callaghan, M. A. F. Barnett, C. E. Adams, J. Henderson, P. Marshall, and others.
44. THE COMMERCIAL RIPENING OF BANANAS IN NEW ZEALAND. J. B. Hyatt and C. W O. Turner. Price, 1s.
45. GOITRE IN SCHOOL-CHILDREN. R. A. Shore and R. L. Andrew. Price ,1s. 6d.
46. " CROSS 7 " WHEAT. O. H. Frankel. Price, 1s.
47. THE GRASSLANDS OF THE SOUTH ISLAND OF NEW ZEALAND. (Out of print.)
48. WATER-SUPPLIES OF FARMS AND DAIRY FACTORIES IN HAMILTON BASIN AND HAURAKI LOWLAND. N. H. Taylor. Price, 1s. 6d.
49. INTRODUCTION TO THE GRASSES OF NEW ZEALAND. (Out of print.) H. H. Allan.
50. THE CHEESE YIELDING CAPACITY OF MILK. F. H. McDowall. Price, 3s. 6d. (Obtainable from the Dairy Research Institute, Massey College, Palmerston North.)
51. THE CHILLING OF BEEF FOR EXPORT. C. R. Barnicoat. Price, 1s. 3d.
52. INVESTIGATION OF FEED FLAVOUR IN CREAM AND BUTTER. W. Riddet, E. Bruce Levy, and others. Price, 1s.
53. A PRELIMINARY SURVEY OF THE CITRUS INDUSTRY IN NEW ZEALAND.
54. THE LITTLE BARRIER ISLAND. (Out of print.)
55. THE WEST NELSON EARTHQUAKES OF 1929.
56. WHEAT RESEARCH INSTITUTE : FOURTH ANNUAL REPORT. (Out of print.)
57. REPORT OF INTERDEPARTMENTAL COMMITTEE ON SILICOSIS. Price, 1s. 3d.
58. FARM MANAGEMENT SURVEY, PLAINS AREA ASHBURTON COUNTY, NEW ZEALAND. (Out of print.)

BULLETINS OF THE DEPARTMENT OF SCIENTIFIC AND INDUSTRIAL RESEARCH—*continued*

No.

59. THE MANUFACTURE OF SODIUM CHLORATE FOR WEED-KILLING : A POSSIBLE ELECTRO CHEMICAL INDUSTRY FOR NEW ZEALAND. (Out of print.) S. H. Wilson.
60. PETROLEUM IN NEW ZEALAND. J. Henderson. Price, 1s.
61. THE SOILS AND AGRICULTURE OF WESTERN SAMOA. W. M. Hamilton and L. I. Grange. Price, 1s
62. LAND DETERIORATION IN THE HEAVIER RAINFALL DISTRICTS OF NEW ZEALAND.
63. REPORT ON THE ORGANIZATION OF ANIMAL RESEARCH IN NEW ZEALAND. J. Hammond. Price, 1s.
64. GENETICS AND LIVE-STOCK PRODUCTION. (Out of print.) F. W. Dry.
65. SOIL SURVEY OF DUVAUCHELLE BAY-WAINUI DISTRICT BANKS PENINSULA. C. S. Harris and A. C. Harris. Price, 1s. 3d.
66. A SURVEY OF TUNG GROVES IN NEW ZEALAND. M. M. Burns and N. H. Taylor. Price, 2s.
67. A LIST OF PLANT DISEASES RECORDED IN NEW ZEALAND. R. M. Brien. Price, 1s.
68. WHEAT RESEARCH INSTITUTE : FIFTH ANNUAL REPORT. Price, 1s.
69. SCIENTIFIC AND INDUSTRIAL RESEARCH, 1927-38. Price, 2s. 6d.
70. LAND UTILIZATION REPORT OF THE HERETAUNGA PLAINS. (Out of print.)
71. SOIL SURVEY OF WESTPORT DISTRICT. C. S. Harris and A. C. Harris. Price, 1s. 6d.
72. SOIL SURVEY OF WAIRAU PLAINS, MARLBOROUGH. C. S. Harris and K. S. Birrell. Price, 2s.
73. SURVEY OF THE TUSSOCK-GRASSLANDS OF THE SOUTH ISLAND, NEW ZEALAND. V. D. Zotov Price, 1s.
74. SOME GROUND-WAVE FIELD-INTENSITY MEASUREMENTS TAKEN IN NEW ZEALAND. G. Searle. Price, 1s.
75. A SURVEY OF THE STANDARDS OF LIFE OF NEW ZEALAND DAIRY-FARMERS. W T. Doig. Price, 2s. 6d.
76. SOILS AND AGRICULTURE OF PART OF WAIPA COUNTY. (Out of print.)
77. MAINTENANCE OF VEGETATION COVER IN NEW ZEALAND, WITH SPECIAL REFERENCE TO LAND EROSION. (Out of print.)
78. DIAMOND-BACK MOTH INVESTIGATION IN NEW ZEALAND. (Out of print.)
79. THE GRASSLANDS OF THE NORTH ISLAND OF NEW ZEALAND. (Out of print.)
80. THE CURING AND COLOURING OF NEW ZEALAND LEMONS. J. B. Hyatt and O. H. Keys. Price, 1s.
81. WHEAT RESEARCH INSTITUTE : SIXTH ANNUAL REPORT. (Out of print.) Price, 1s.
82. BOTANICAL ASPECTS OF RAGWORT (*SENECIO JACOBAEA* L.) CONTROL. A. L. Poole and D. Cairns. Price, 2s. 6d.
83. A HANDBOOK OF THE NATURALIZED FLORA OF NEW ZEALAND. H. H. Allan. (Out of print. Price, 10s and 10s. 6d.
84. WHEAT RESEARCH INSTITUTE : SEVENTH ANNUAL REPORT. Price, 1s.
85. THE ECONOMIC IMPORTANCE OF SEAWEEDS. (Out of print.) L. B. Moore.
86. MOLE DRAINAGE IN NEW ZEALAND.
87. WHEAT RESEARCH INSTITUTE : EIGHTH ANNUAL REPORT. (Out of print.)
88. FIRECLAY AND GANISTER IN NEW ZEALAND. J. Henderson. Price, 2s. 6d.
89. THE DAIRY INDUSTRY IN NEW ZEALAND. W. M. Hamilton. Price, 2s. 6d.
90. MILBURN-CLARENDON PHOSPHATE DEPOSITS. E. O. Macpherson. Price, 2s. 5d.
91. NASSELLA TUSSOCK. A. J. Healy. Price, 2s. 9d.
92. SOIL EROSION IN THE HIGH COUNTRY OF THE SOUTH ISLAND H. S. Gibbs, J. D. Raeside, J. K. Dixon, and A. J. Metson. Price, 5s.
93. INVESTIGATION OF THE CLARENDON PHOSPHATE DEPOSITS. R. W. Willett. Price, 5s.
94. SOILS AND SOME RELATED ASPECTS OF AGRICULTURE IN MID HAWKE'S BAY. I. J. Pohlen and others. Price, 5s.
95. SOIL EROSION IN NEW ZEALAND—PART 1 : SOUTHERN HALF OF NORTH ISLAND. L. I. Grange and H. S. Gibbs. Price, 2s. 6d.
96. RHYTHMS IN NEMATODE PARASITISM OF SHEEP J. H. Tetley. Price, 8s. 6d.

ALL PRICES POST FREE

THE NEW ZEALAND JOURNAL OF SCIENCE AND TECHNOLOGY. [Vols. 1-29.]

Vol. 30 now appearing.

ANNUAL SUBSCRIPTION—Volume 30. Section A (Agricultural Research Section), 10s. Section B (General Research Section), 10s. Sections A and B, 15s. Single copy, 2s.: Postage 1d. (See inside front cover).

Order through your Bookseller or from

THE PUBLICATIONS OFFICER, DEPARTMENT OF SCIENTIFIC AND INDUSTRIAL RESEARCH, P.O. BOX 562, DUNEDIN

and from

THE HIGH COMMISSIONER FOR N.Z., 415 STRAND, LONDON, W.C.2

All Orders should be accompanied by remittance. To country cheques add exchange (6d.)

GOULLS SOMERVILLE WILKIE LTD.

1,500 10/50